

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C12N 15/00</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 00/31247</b> <b>(43) International Publication Date:</b> 2 June 2000 (02.06.00)
<b>(21) International Application Number:</b> PCT/US99/27438 <b>(22) International Filing Date:</b> 19 November 1999 (19.11.99)  <b>(30) Priority Data:</b> 60/109,401 20 November 1998 (20.11.98) US 60/119,386 10 February 1999 (10.02.99) US 60/122,620 3 March 1999 (03.03.99) US 60/130,560 22 April 1999 (22.04.99) US  <b>(71) Applicant:</b> KOSAN BIOSCIENCES, INC. [US/US]; 3832 Bay Center Drive, Hayward, CA 94545 (US).  <b>(72) Inventors:</b> JULIEN, Bryan; 4514 Fleming Avenue, Oakland, CA 94619 (US). KATZ, Leonard; 22294 City Center Drive, Apartment 5104, Hayward, CA 95051 (US). KHOSLA, Chaitan; 740 La Para Avenue, Palo Alto, CA 94306 (US). TANG, Li; 574 Cutwater Lane, Foster City, CA 94404 (US). ZIERMANN, Rainer; 3908 Leona Street, San Mateo, CA 94403 (US).  <b>(74) Agents:</b> MURASHIGE, Kate, H. et al.; Morrison & Foerster LLP, 2000 Pennsylvania Avenue, N.W., Washington, DC 20006-1888 (US).		<b>(81) Designated States:</b> AL, AM, AU, BA, BB, BG, BR, CA, CN, CR, CU, CZ, DM, EE, GD, GE, HR, HU, IL, IS, JP, KG, KP, KR, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, UZ, VN, ZA, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> RECOMBINANT METHODS AND MATERIALS FOR PRODUCING EPOTHILONE AND EPOTHILONE DERIVATIVES		
<b>(57) Abstract</b>  Recombinant nucleic acids that encode all or a portion of the epothilone polyketide synthase (PKS) are used to express recombinant PKS genes in host cells for the production of epothilones, epothilone derivatives, and polyketides that are useful as cancer chemotherapeutics, fungicides, and immunosuppressants.		

BEST AVAILABLE COPY

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

## RECOMBINANT METHODS AND MATERIALS FOR PRODUCING EPOTHILONE AND EPOTHILONE DERIVATIVES

5

### Reference to Government Funding

This invention was supported in part by SBIR grant 1R43-CA79228-01. The U.S. government has certain rights in this invention.

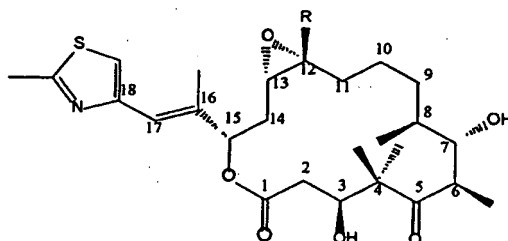
### 10 Field of the Invention

The present invention provides recombinant methods and materials for producing epothilone and epothilone derivatives. The invention relates to the fields of agriculture, chemistry, medicinal chemistry, medicine, molecular biology, and pharmacology.

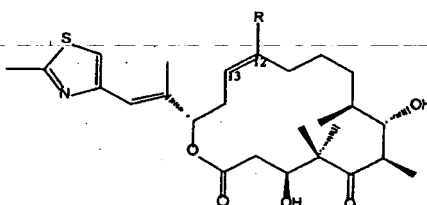
### 15 Background of the Invention

The epothilones were first identified by Gerhard Hofle and colleagues at the National Biotechnology Research Institute as an antifungal activity extracted from the myxobacterium *Sorangium cellulosum* (see K. Gerth *et al.*, 1996, J. Antibiotics 49: 560-563 and Germany Patent No. DE 41 38 042). The epothilones were later found to have  
20 activity in a tubulin polymerization assay (see D. Bollag *et al.*, 1995, Cancer Res. 55:2325-2333) to identify antitumor agents and have since been extensively studied as potential antitumor agents for the treatment of cancer.

The chemical structure of the epothilones produced by *Sorangium cellulosum* strain So ce 90 was described in Hofle *et al.*, 1996, Epothilone A and B - novel 16-  
25 membered macrolides with cytotoxic activity: isolation, crystal structure, and conformation in solution, Angew. Chem. Int. Ed. Engl. 35(13/14): 1567-1569, incorporated herein by reference. The strain was found to produce two epothilone compounds, designated A (R = H) and B (R = CH<sub>3</sub>), as shown below, which showed broad cytotoxic activity against eukaryotic cells and noticeable activity and selectivity against  
30 breast and colon tumor cell lines.



The desoxy counterparts of epothilones A and B, also known as epothilones C ( $R = H$ ) and D ( $R = CH_3$ ), are known to be less cytotoxic, and the structures of these epothilones are shown below.



Two other naturally occurring epothilones have been described. These are epothilones E and F, in which the methyl side chain of the thiazole moiety of epothilones A and B has been hydroxylated to yield epothilones E and F, respectively.

Because of the potential for use of the epothilones as anticancer agents, and because of the low levels of epothilone produced by the native *Solenastrea* strain, a number of research teams undertook the effort to synthesize the epothilones. This effort has been successful (see Balog *et al.*, 1996, Total synthesis of (-)-epothilone A, *Angew. Chem. Int. Ed. Engl.* 35(23/24): 2801-2803; Su *et al.*, 1997, Total synthesis of (-)-epothilone B: an extension of the Suzuki coupling method and insights into structure-activity relationships of the epothilones, *Angew. Chem. Int. Ed. Engl.* 36(7): 757-759; Meng *et al.*, 1997, Total syntheses of epothilones A and B, *JACS* 119(42): 10073-10092; and Balog *et al.*, 1998, A novel aldol condensation with 2-methyl-4-pentenol and its application to an improved total synthesis of epothilone B, *Angew. Chem. Int. Ed. Engl.* 37(19): 2675-2678, each of which is incorporated herein by reference). Despite the success of these efforts, the chemical synthesis of the epothilones is tedious, time-consuming, and expensive. Indeed, the methods have been characterized as impractical for the full-scale pharmaceutical development of an epothilone.

A number of epothilone derivatives, as well as epothilones A - D, have been studied *in vitro* and *in vivo* (see Su *et al.*, 1997, Structure-activity relationships of the epothilones and the first *in vivo* comparison with paclitaxel, *Angew. Chem. Int. Ed. Engl.*

36(19): 2093-2096; and Chou *et al.*, Aug. 1998, Desoxyepothilone B: an efficacious microtubule-targeted antitumor agent with a promising *in vivo* profile relative to epothilone B, Proc. Natl. Acad. Sci. USA 95: 9642-9647, each of which is incorporated herein by reference). Additional epothilone derivatives and methods for synthesizing epothilones and epothilone derivatives are described in PCT patent publication Nos. 5 99/54330, 99/54319, 99/54318, 99/43653, 99/43320, 99/42602, 99/40047, 99/27890, 99/07692, 99/02514, 99/01124, 98/25929, 98/22461, 98/08849, and 97/19086; U.S. Patent No. 5,969,145; and Germany patent publication No. DE 41 38 042, each of which is incorporated herein by reference.

10        There remains a need for economical means to produce not only the naturally occurring epothilones but also the derivatives or precursors thereof, as well as new epothilone derivatives with improved properties. There remains a need for a host cell that produces epothilones or epothilone derivatives that is easier to manipulate and ferment than the natural producer *Sorangium cellulosum*. The present invention meets these and 15 other needs.

#### Summary of the Invention

In one embodiment, the present invention provides recombinant DNA compounds that encode the proteins required to produce epothilones A, B, C, and D. The present 20 invention also provides recombinant DNA compounds that encode portions of these proteins. The present invention also provides recombinant DNA compounds that encode a hybrid protein, which hybrid protein includes all or a portion of a protein involved in epothilone biosynthesis and all or a portion of a protein involved in the biosynthesis of another polyketide or non-ribosomal-derived peptide. In a preferred embodiment, the 25 recombinant DNA compounds of the invention are recombinant DNA cloning vectors that facilitate manipulation of the coding sequences or recombinant DNA expression vectors that code for the expression of one or more of the proteins of the invention in recombinant host cells.

In another embodiment, the present invention provides recombinant host cells that 30 produce a desired epothilone or epothilone derivative. In one embodiment, the invention provides host cells that produce one or more of the epothilones or epothilone derivatives at higher levels than produced in the naturally occurring organisms that produce epothilones. In another embodiment, the invention provides host cells that produce mixtures of

epothilones that are less complex than the mixtures produced by naturally occurring host cells. In another embodiment, the present invention provides non-*Sorangium* recombinant host cells that produce an epothilone or epothilone derivative.

In a preferred embodiment, the host cells of the invention produce less complex mixtures of epothilones than do naturally occurring cells that produce epothilones. Naturally occurring cells that produce epothilones typically produce a mixture of epothilones A, B, C, D, E, and F. The table below summarizes the epothilones produced in different illustrative host cells of the invention.

<u>Cell Type</u>	<u>Epothilones Produced</u>	<u>Epothilones Not Produced</u>
1	A, B, C, D, E, F	-----
2	A, C, E	B, D, F
3	B, D, F	A, C, E
4	A, B, C, D	E, F
5	A, C	B, D, E, F
6	C	A, B, D, E, F
7	B, D	A, C, E, F
8	D	A, B, C, E, F

In addition, cell types may be constructed which produce only the newly discovered epothilones G and H, further discussed below, and one or the other of G and H or both in combination with the downstream epothilones. Thus, it is understood, based on the present invention, that the biosynthetic pathway which relates the naturally occurring epothilones is, respectively,  $G \rightarrow C \rightarrow A \rightarrow E$  and  $H \rightarrow D \rightarrow B \rightarrow F$ . Appropriate enzymes may also convert members of each pathway to the corresponding member of the other.

Thus, the recombinant host cells of the invention also include host cells that produce only one desired epothilone or epothilone derivative.

In another embodiment, the invention provides *Sorangium* host cells that have been modified genetically to produce epothilones either at levels greater than those observed in naturally occurring host cells or as less complex mixtures of epothilones than produced by naturally occurring host cells, or produce an epothilone derivative that is not produced in nature. In a preferred embodiment, the host cell produces the epothilones at equal to or greater than 20 mg/L.

In another embodiment, the recombinant host cells of the invention are host cells other than *Sorangium cellulosum* that have been modified genetically to produce an epothilone or an epothilone derivative. In a preferred embodiment, the host cell produces the epothilones at equal to or greater than 20 mg/L. In a more preferred embodiment, the recombinant host cells are *Myxococcus*, *Pseudomonas*, or *Streptomyces* host cells that produce the epothilones or an epothilone derivative at equal to or greater than 20 mg/L. In another embodiment, the present invention provides novel compounds useful in agriculture, veterinary practice, and medicine. In one embodiment, the compounds are useful as fungicides. In another embodiment, the compounds are useful in cancer chemotherapy. In a preferred embodiment, the compound is an epothilone derivative that is at least as potent against tumor cells as epothilone B or D. In another embodiment, the compounds are useful as immunosuppressants. In another embodiment, the compounds are useful in the manufacture of another compound. In a preferred embodiment, the compounds are formulated in a mixture or solution for administration to a human or animal.

These and other embodiments of the invention are described in more detail in the following description, the examples, and claims set forth below.

#### Brief Description of the Figures

Figure 1 shows a restriction site map of the insert *Sorangium cellulosum* genomic DNA in four overlapping cosmid clones (designated 8A3, 1A2, 4, and 85 and corresponding to pKOS35-70.8A3, pKOS35-70.1A2, pKOS35-70.4, and pKOS35-79.85, respectively) spanning the epothilone gene cluster. A functional map of the epothilone gene cluster is also shown. The loading domain (Loading, *epoA*), the non-ribosomal peptide synthase (NRPS, Module 1, *epoB*) module, and each module (Modules 2 through 9, *epoC*, *epoD*, *epoE*, and *epoF*) of the remaining eight modules of the epothilone synthase gene are shown, as is the location of the *epoK* gene that encodes a cytochrome P450-like epoxidation enzyme.

Figure 2 shows a number of precursor compounds to N-acylcysteamine thioester derivatives that can be supplied to an epothilone PKS of the invention in which the NRPS-like module 1 or module 2 KS domain has been inactivated to produce a novel epothilone derivative. A general synthetic procedure for making such compounds is also shown.

Figure 3 shows restriction site and function maps of plasmids pKOS35-82.1 and pKOS35-82.2.

Figure 4 shows restriction site and function maps of plasmids pKOS35-154 and pKOS90-22.

5        Figure 5 shows a schematic of a protocol for introducing the epothilone PKS and modification enzyme genes into the chromosome of a *Myxococcus xanthus* host cell as described in Example 3.

Figure 6 shows restriction site and function maps of plasmids pKOS039-124 and pKOS039-124R.

10       Figure 7 shows a restriction site and function map of plasmid pKOS039-126R.

Figure 8 shows a restriction site and function map of plasmid pKOS039-141.

Figure 9 shows a restriction site and function map of plasmid pKOS045-12.

#### Detailed Description of the Invention

15        The present invention provides the genes and proteins that synthesize the epothilones in *Sorangium cellulosum* in recombinant and isolated form. As used herein, the term recombinant refers to a compound or composition produced by human intervention, typically by specific and directed manipulation of a gene or portion thereof. The term isolated refers to a compound or composition in a preparation that is

20       substantially free of contaminating or undesired materials or, with respect to a compound or composition found in nature, substantially free of the materials with which that compound or composition is associated in its natural state. The epothilones (epothilone A, B, C, D, E, and F) and compounds structurally related thereto (epothilone derivatives) are potent cytotoxic agents specific for eukaryotic cells. These compounds have application as

25       anti-fungals, cancer chemotherapeutics, and immunosuppressants. The epothilones are produced at very low levels in the naturally occurring *Sorangium cellulosum* cells in which they have been identified. Moreover, *S. cellulosum* is very slow growing, and fermentation of *S. cellulosum* strains is difficult and time-consuming. One important benefit conferred by the present invention is the ability simply to produce an epothilone or

30       epothilone derivative in a non-*S. cellulosum* host cell. Another advantage of the present invention is the ability to produce the epothilones at higher levels and in greater amounts in the recombinant host cells provided by the invention than possible in the naturally



occurring epothilone producer cells. Yet another advantage is the ability to produce an epothilone derivative in a recombinant host cell.

The isolation of recombinant DNA encoding the epothilone biosynthetic genes resulted from the probing of a genomic library of *Sorangium cellulosum* SMP44 DNA. As described more fully in Example 1 below, the library was prepared by partially digesting *S. cellulosum* genomic DNA with restriction enzyme *Sau*IIIA1 and inserting the DNA fragments generated into *Bam*HI-digested Supercos™ cosmid DNA (Stratagene). Cosmid clones containing epothilone gene sequences were identified by probing with DNA probes specific for sequences from PKS genes and reprobing with secondary probes comprising nucleotide sequences identified with the primary probes.

Four overlapping cosmid clones were identified by this effort. These four cosmids were deposited with the American Type Culture Collection (ATCC), Manassas, VA, USA, under the terms of the Budapest Treaty, and assigned ATCC accession numbers. The clones (and accession numbers) were designated as cosmids pKOS35-70.1A2 (ATCC 203782), pKOS35-70.4 (ATCC 203781), pKOS35-70.8A3 (ATCC 203783), and pKOS35-79.85 (ATCC 203780). The cosmids contain insert DNA that completely spans the epothilone gene cluster. A restriction site map of these cosmids is shown in Figure 1. Figure 1 also provides a function map of the epothilone gene cluster, showing the location of the six epothilone PKS genes and the *epoK* P450 epoxidase gene.

The epothilone PKS genes, like other PKS genes, are composed of coding sequences organized to encode a loading domain, a number of modules, and a thioesterase domain. As described more fully below, each of these domains and modules corresponds to a polypeptide with one or more specific functions. Generally, the loading domain is responsible for binding the first building block used to synthesize the polyketide and transferring it to the first module. The building blocks used to form complex polyketides are typically acylthioesters, most commonly acetyl, propionyl, malonyl, methylmalonyl, and ethylmalonyl CoA. Other building blocks include amino acid-like acylthioesters. PKSs catalyze the biosynthesis of polyketides through repeated, decarboxylative Claisen condensations between the acylthioester building blocks. Each module is responsible for binding a building block, performing one or more functions on that building block, and transferring the resulting compound to the next module. The next module, in turn, is responsible for attaching the next building block and transferring the growing compound

to the next module until synthesis is complete. At that point, an enzymatic thioesterase (TE) activity cleaves the polyketide from the PKS.

Such modular organization is characteristic of the class of PKS enzymes that synthesize complex polyketides and is well known in the art. Recombinant methods for manipulating modular PKS genes are described in U.S. Patent Nos. 5,672,491; 5,712,146; 5,830,750; and 5,843,718; and in PCT patent publication Nos. 98/49315 and 97/02358, each of which is incorporated herein by reference. The polyketide known as 6-deoxyerythronolide B (6-dEB) is synthesized by a PKS that is a prototypical modular PKS enzyme. The genes, known as *eryAI*, *eryAII*, and *eryAIII*, that code for the multi-subunit protein known as deoxyerythronolide B synthase or DEBS (each subunit is known as DEBS1, DEBS2, or DEBS3) that synthesizes 6-dEB are described in U.S. Patent Nos. 5,712,146 and 5,824,513, incorporated herein by reference.

The loading domain of the DEBS PKS consists of an acyltransferase (AT) and an acyl carrier protein (ACP). The AT of the DEBS loading domain recognizes propionyl CoA (other loading domain ATs can recognize other acyl-CoAs, such as acetyl, malonyl, methylmalonyl, or butyryl CoA) and transfers it as a thioester to the ACP of the loading domain. Concurrently, the AT on each of the six extender modules recognizes a methylmalonyl CoA (other extender module ATs can recognize other CoAs, such as malonyl or alpha-substituted malonyl CoAs, i.e., malonyl, ethylmalonyl, and 2-hydroxymalonyl CoA) and transfers it to the ACP of that module to form a thioester. Once DEBS is primed with acyl- and methylmalonyl-ACPs, the acyl group of the loading domain migrates to form a thioester (trans-esterification) at the KS of the first module; at this stage, module one possesses an acyl-KS adjacent to a methylmalonyl ACP. The acyl group derived from the DEBS loading domain is then covalently attached to the alpha-carbon of the extender group to form a carbon-carbon bond, driven by concomitant decarboxylation, and generating a new acyl-ACP that has a backbone two carbons longer than the loading unit (elongation or extension). The growing polyketide chain is transferred from the ACP to the KS of the next module of DEBS, and the process continues.

The polyketide chain, growing by two carbons for each module of DEBS, is sequentially passed as a covalently bound thioester from module to module, in an assembly line-like process. The carbon chain produced by this process alone would possess a ketone at every other carbon atom, producing a polyketone, from which the

name polyketide arises. Commonly, however, additional enzymatic activities modify the beta keto group of each two carbon unit just after it has been added to the growing polyketide chain but before it is transferred to the next module. Thus, in addition to the minimal module containing KS, AT, and ACP necessary to form the carbon-carbon bond, modules may contain a ketoreductase (KR) that reduces the keto group to an alcohol. Modules may also contain a KR plus a dehydratase (DH) that dehydrates the alcohol to a double bond. Modules may also contain a KR, a DH, and an enoylreductase (ER) that converts the double bond to a saturated single bond using the beta carbon as a methylene function. The DEBS modules include those with only a KR domain, only an inactive KR domain, and with all three KR, DH, and ER domains.

Once a polyketide chain traverses the final module of a PKS, it encounters the releasing domain or thioesterase found at the carboxyl end of most PKSs. Here, the polyketide is cleaved from the enzyme and, for most but not all polyketides, cyclized. The polyketide can be modified further by tailoring or modification enzymes; these enzymes add carbohydrate groups or methyl groups, or make other modifications, i.e., oxidation or reduction, on the polyketide core molecule. For example, 6-dEB is hydroxylated, methylated, and glycosylated (glycosidated) to yield the well known antibiotic erythromycin A in the *Saccharopolyspora erythraea* cells in which it is produced naturally.

While the above description applies generally to modular PKS enzymes and specifically to DEBS, there are a number of variations that exist in nature. For example, many PKS enzymes comprise loading domains that, unlike the loading domain of DEBS, comprise an "inactive" KS domain that functions as a decarboxylase. This inactive KS is in most instances called  $KS^Q$ , where the superscript is the single-letter abbreviation for the amino acid (glutamine) that is present instead of the active site cysteine required for ketosynthase activity. The epothilone PKS loading domain contains a  $KS^Y$  domain not present in other PKS enzymes for which amino acid sequence is currently available in which the amino acid tyrosine has replaced the cysteine. The present invention provides recombinant DNA coding sequences for this novel KS domain.

Another important variation in PKS enzymes relates to the type of building block incorporated. Some polyketides, including epothilone, incorporate an amino acid derived building block. PKS enzymes that make such polyketides require specialized modules for incorporation. Such modules are called non-ribosomal peptide synthetase (NRPS)

modules. The epothilone PKS, for example, contains an NRPS module. Another example of a variation relates to additional activities in a module. For example, one module of the epothilone PKS contains a methyltransferase (MT) domain, a heretofore unknown domain of PKS enzymes that make modular polyketides.

5       The complete nucleotide sequence of the coding sequence of the open reading frames (ORFs) of the epothilone PKS genes and epothilone tailoring (modification) enzyme genes is provided in Example 1, below. This sequence information together with the information provided below regarding the locations of the open reading frames of the genes within that sequence provides the amino acid sequence of the encoded proteins.

10       Those of skill in the art will recognize that, due to the degenerate nature of the genetic code, a variety of DNA compounds differing in their nucleotide sequences can be used to encode a given amino acid sequence of the invention. The native DNA sequence encoding the epothilone PKS and epothilone modification enzymes of *Sorangium cellulosum* is shown herein merely to illustrate a preferred embodiment of the invention. The present  
15       invention includes DNA compounds of any sequence that encode the amino acid sequences of the polypeptides and proteins of the invention. In similar fashion, a polypeptide can typically tolerate one or more amino acid substitutions, deletions, and insertions in its amino acid sequence without loss or significant loss of a desired activity and, in some instances, even an improvement of a desired activity. The present invention  
20       includes such polypeptides with alternate amino acid sequences, and the amino acid sequences shown merely illustrate preferred embodiments of the invention.

      The present invention provides recombinant genes for the production of epothilones. The invention is exemplified by the cloning, characterization, and manipulation of the epothilone PKS and modification enzymes of *Sorangium cellulosum*  
25       SMP44. The description of the invention and the recombinant vectors deposited in connection with that description enable the identification, cloning, and manipulation of epothilone PKS and modification enzymes from any naturally occurring host cell that produces an epothilone. Such host cells include other *S. cellulosum* strains, such as So ce 90, other *Sorangium* species, and non-*Sorangium* cells. Such identification, cloning, and  
30       characterization can be conducted by those of ordinary skill in accordance with the present invention using standard methodology for identifying homologous DNA sequences and for identifying genes that encode a protein of function similar to a known protein. Moreover, the present invention provides recombinant epothilone PKS and modification

enzyme genes that are synthesized de novo or are assembled from non-epothilone PKS genes to provide an ordered array of domains and modules in one or more proteins that assemble to form a PKS that produces epothilone or an epothilone derivative.

5 The recombinant nucleic acids, proteins, and peptides of the invention are many and diverse. To facilitate an understanding of the invention and the diverse compounds and methods provided thereby, the following discussion describes various regions of the epothilone PKS and corresponding coding sequences. This discussion begins with a general discussion of the genes that encode the PKS, the location of the various domains and modules in those genes, and the location of the various domains in those modules.  
10 Then, a more detailed discussion follows, focusing first on the loading domain, followed by the NRPS module, and then the remaining eight modules of the epothilone PKS.

There are six epothilone PKS genes. The *epoA* gene encodes the 149 kDa loading domain (which can also be referred to as a loading module). The *epoB* gene encodes module 1, the 158 kDa NRPS module. The *epoC* gene encodes the 193 kDa module 2. The  
15 *epoD* gene encodes a 765 kDa protein that comprises modules 3 through 6, inclusive. The *epoE* gene encodes a 405 kDa protein that comprises modules 7 and 8. The *epoF* gene encodes a 257 kDa protein that comprises module 9 and the thioesterase domain. Immediately downstream of the *epoF* gene is *epoK*, the P450 epoxidase gene which encodes a 47 kDa protein, followed immediately by the *epoL* gene, which may encode a  
20 24 kDa dehydratase. The *epoL* gene is followed by a number of ORFs that include genes believed to encode proteins involved in transport and regulation.

The sequences of these genes are shown in Example 1 in one contiguous sequence or contig of 71,989 nucleotides. This contig also contains two genes that appear to originate from a transposon and are identified below as ORF A and ORF B. These two  
25 genes are believed not to be involved in epothilone biosynthesis but could possibly contain sequences that function as a promoter or enhancer. The contig also contains more than 12 additional ORFs, only 12 of which, designated ORF2 through ORF12 and ORF2 complement, are identified below. As noted, ORF2 actually is two ORFs, because the complement of the strand shown also comprises an ORF. The function of the  
30 corresponding gene product, if any, of these ORFs has not yet been established. The Table below provides the location of various open reading frames, module-coding sequences, and domain encoding sequences within the contig sequence shown in Example 1. Those of skill in the art will recognize, upon consideration of the sequence shown in Example 1,

that the actual start locations of several of the genes could differ from the start locations shown in the table, because of the presence in frame codons for methionine or valine in close proximity to the codon indicated as the start codon. The actual start codon can be confirmed by amino acid sequencing of the proteins expressed from the genes.

<u>Start</u>	<u>Stop</u>	<u>Comment</u>
3	992	transposase gene ORF A, not part of the PKS
989	1501	transposase gene ORF B, not part of the PKS
1998	6263	<i>epoA</i> gene, encodes the loading domain
2031	3548	KS <sup>Y</sup> of the loading domain
3621	4661	AT of the loading domain
4917	5810	ER of the loading domain, potentially involved in formation of the thiazole moiety
5856	6155	ACP of the loading domain
6260	10493	<i>epoB</i> gene, encodes module 1, the NRPS module
6620	6649	condensation domain C2 of the NRPS module
6861	6887	heterocyclization signature sequence
6962	6982	condensation domain C4 of the NRPS module
7358	7366	condensation domain C7 (partial) of the NRPS module
7898	7921	adenylation domain A1 of the NRPS module
8261	8308	adenylation domain A3 of the NRPS module
8411	8422	adenylation domain A4 of the NRPS module
8861	8905	adenylation domain A6 of the NRPS module
8966	8983	adenylation domain A7 of the NRPS module
9090	9179	adenylation domain A8 of the NRPS module
9183	9992	oxidation region for forming thiazole
10121	10138	Adenylation domain A10 of the NRPS module
10261	10306	Thiolation domain (PCP) of the NRPS module
10639	16137	<i>epoC</i> gene, encodes module 2
10654	12033	KS2, the KS domain of module 2
12250	13287	AT2, the AT domain of module 2
13327	13899	DH2, the DH domain of module 2
14962	15756	KR2, the KR domain of module 2
15763	16008	ACP2, the ACP domain of module 2
16134	37907	<i>epoD</i> gene, encodes modules 3-6
16425	17606	KS3
17817	18857	AT3

<u>Start</u>	<u>Stop</u>	<u>Comment</u>
19581	20396	KR3
20424	20642	ACP3
20706	22082	KS4
22296	23336	AT4
24069	24647	KR4
24867	25151	ACP4
25203	26576	KS5
26793	27833	AT5
27966	28574	DH5
29433	30287	ER5
30321	30869	KR5
31077	31373	ACP5
31440	32807	KS6
33018	34067	AT6
34107	34676	DH6
35760	36641	ER6
36705	37256	KR6
37470	37769	ACP6
37912	49308	<i>epoE</i> gene, encodes modules 7 and 8
38014	39375	KS7
39589	40626	AT7
41341	41922	KR7
42181	42423	ACP7
42478	43851	KS8
44065	45102	AT8
45262	45810	DH (inactive)
46072	47172	MT8, the methyltransferase domain of module 8
48103	48636	KR8, this domain is inactive
48850	49149	ACP8
49323	56642	<i>epoF</i> gene, encodes module 9 and the TE domain
49416	50774	KS9
50985	52025	AT9
52173	53414	DH (inactive)
54747	55313	KR9
55593	55805	ACP9
55878	56600	TE9, the thioesterase domain
56757	58016	<i>epoK</i> gene, encodes the P450 epoxidase

<u>Start</u>	<u>Stop</u>	<u>Comment</u>
58194	58733	<i>epoL</i> gene (putative dehydratase)
59405	59974	ORF2 complement, complement of strand shown
59460	60249	ORF2
60271	60738	ORF3, complement of strand shown
61730	62647	ORF4 (putative transporter)
63725	64333	ORF5
64372	65643	ORF6
66237	67472	ORF7 (putative oxidoreductase)
67572	68837	ORF8 (putative oxidoreductase membrane subunit)
68837	69373	ORF9
69993	71174	ORF10 (putative transporter)
71171	71542	ORF11
71557	71989	ORF12

With this overview of the organization and sequence of the epothilone gene cluster, one can better appreciate the many different recombinant DNA compounds provided by the present invention.

- 5        The epothilone PKS is multiprotein complex composed of the gene products of the *epoA*, *epoB*, *epoC*, *epoD*, *epoE*, and *epoF* genes. To confer the ability to produce epothilones to a host cell, one provides the host cell with the recombinant *epoA*, *epoB*, *epoC*, *epoD*, *epoE*, and *epoF* genes of the present invention, and optionally other genes, capable of expression in that host cell. Those of skill in the art will appreciate that, while
- 10    the epothilone and other PKS enzymes may be referred to as a single entity herein, these enzymes are typically multisubunit proteins. Thus, one can make a derivative PKS (a PKS that differs from a naturally occurring PKS by deletion or mutation) or hybrid PKS (a PKS that is composed of portions of two different PKS enzymes) by altering one or more genes that encode one or more of the multiple proteins that constitute the PKS.
- 15        The post-PKS modification or tailoring of epothilone includes multiple steps mediated by multiple enzymes. These enzymes are referred to herein as tailoring or modification enzymes. Surprisingly, the products of the domains of the epothilone PKS predicted to be functional by analysis of the genes that encode them are compounds that have not been previously reported. These compounds are referred to herein as epothilones
- 20    G and H. Epothilones G and H lack the C-12-C-13  $\pi$ -bond of epothilones C and D and the C-12-C-13 epoxide of epothilones A and B, having instead a hydrogen and hydroxyl



group at C-13, a single bond between C-12 and C-13, and a hydrogen and H or methyl group at C-12. These compounds are predicted to result from the epothilone PKS, because the DNA and corresponding amino acid sequence for module 4 of the epothilone PKS does not appear to include a DH domain.

5 As described below, however, expression of the epothilone PKS genes *epoA*, *epoB*, *epoC*, *epoD*, *epoE*, and *epoF* in certain heterologous host cells that do not express *epoK* or *epoL* leads to the production of epothilones C and D, which lack the C-13 hydroxyl and have a double bond between C-12 and C-13. The dehydration reaction that mediates the formation of this double bond may be due to the action of an as yet unrecognized domain  
10 of the epothilone PKS (for example, dehydration could occur in the next module, which possesses an active DH domain and could generate a conjugated diene precursor prior to its dehydrogenation by an ER domain) or an endogenous enzyme in the heterologous host cells (*Streptomyces coelicolor*) in which it was observed. In the latter event, epothilones G and H may be produced in *Sorangium cellulosum* or other host cells and, to be converted  
15 to epothilones C and D, by the action of a dehydratase, which may be encoded by the *epoL* gene. In any event, epothilones C and D are converted to epothilones A and B by an epoxidase encoded by the *epoK* gene. Epothilones A and B are converted to epothilones E and F by a hydroxylase gene, which may be encoded by one of the ORFs identified above or by another gene endogenous to *Sorangium cellulosum*. Thus, one can produce an  
20 epothilone or epothilone derivative modified as desired in a host cell by providing that host cell with one or more of the recombinant modification enzyme genes provided by the invention or by utilizing a host cell that naturally expresses (or does not express) the modification enzyme. Thus, in general, by utilizing the appropriate host and by appropriate inactivation, if desired, of modification enzymes, one may interrupt the  
25 progression of  $G \rightarrow C \rightarrow A \rightarrow E$  or the corresponding downstream processing of epothilone H at any desired point; by controlling methylation, one or both of the pathways can be selected.

Thus, the present invention provides a wide variety of recombinant DNA compounds and host cells for expressing the naturally occurring epothilones A, B, C, and  
30 D and derivatives thereof. The invention also provides recombinant host cells, particularly *Sorangium cellulosum* host cells that produce epothilone derivatives modified in a manner similar to epothilones E and F. Moreover, the invention provides host cells that can produce the heretofore unknown epothilones G and H, either by expression of the

epothilone PKS genes in host cells that do not express the dehydratase that converts epothilones G and H to C and D or by mutating or altering the PKS to abolish the dehydratase function, if it is present in the epothilone PKS.

5 The macrolide compounds that are products of the PKS cluster can thus be modified in various ways. In addition to the modifications described above, the PKS products can be glycosylated, hydroxylated, dehydroxylated, oxidized, methylated and demethylated using appropriate enzymes. Thus, in addition to modifying the product of the PKS cluster by altering the number, functionality, or specificity of the modules contained in the PKS, additional compounds within the scope of the invention can be  
10 produced by additional enzyme-catalyzed activity either provided by a host cell in which the polyketide synthases are produced or by modifying these cells to contain additional enzymes or by additional *in vitro* modification using purified enzymes or crude extracts or, indeed, by chemical modification.

The present invention also provides a wide variety of recombinant DNA  
15 compounds and host cells that make epothilone derivatives. As used herein, the phrase "epothilone derivative" refers to a compound that is produced by a recombinant epothilone PKS in which at least one domain has been either rendered inactive, mutated to alter its catalytic function, or replaced by a domain with a different function or in which a domain has been inserted. In any event, the "epothilone derivative PKS" functions to produce a  
20 compound that differs in structure from a naturally occurring epothilone but retains its ring backbone structure and so is called an "epothilone derivative." To facilitate a better understanding of the recombinant DNA compounds and host cells provided by the invention, a detailed discussion of the loading domain and each of the modules of the epothilone PKS, as well as novel recombinant derivatives thereof, is provided below.

25 The loading domain of the epothilone PKS includes an inactive KS domain, KS<sup>Y</sup>, an AT domain specific for malonyl CoA (which is believed to be decarboxylated by the KS<sup>Y</sup> domain to yield an acetyl group), and an ACP domain. The present invention provides recombinant DNA compounds that encode the epothilone loading domain. The loading domain coding sequence is contained within an ~8.3 kb EcoRI restriction  
30 fragment of cosmid pKOS35-70.8A3. The KS domain is referred to as inactive, because the active site region "TAYSSSL" of the KS domain of the loading domain has a Y residue in place of the cysteine required for ketosynthase activity; this domain does have

decarboxylase activity. See Witkowski *et al.*, 7 Sep. 1999, Biochem. 38(36): 11643-11650, incorporated herein by reference.

The presence of the Y residue in place of a Q residue (which occurs typically in an inactive loading domain KS) may make the KS domain less efficient at decarboxylation.

5 The present invention provides a recombinant epothilone PKS loading domain and corresponding DNA sequences that encode an epothilone PKS loading domain in which the Y residue has been changed to a Q residue by changing the codon therefor in the coding sequence of the loading domain. The present invention also provides recombinant PKS enzymes comprising such loading domains and host cells for producing such

10 enzymes and the polyketides produced thereby. These recombinant loading domains include those in which just the Y residue has been changed, those in which amino acids surrounding and including the Y domain have been changed, and those in which the complete KS<sup>Y</sup> domain has been replaced by a complete KS<sup>Q</sup> domain. The latter embodiment includes but is not limited to a recombinant epothilone loading domain in

15 which the KS<sup>Y</sup> domain has been replaced by the KS<sup>Q</sup> domain of the oleandolide PKS or the narbonolide PKS (see the references cited below in connection with the oleandomycin, narbomycin, and picromycin PKS and modification enzymes).

The epothilone loading domain also contains an AT domain believed to bind malonyl CoA. The sequence "QTAFTQPALFTFEYALAALW. . . GHSIG" in the AT

20 domain is consistent with malonyl CoA specificity. As noted above, the malonyl CoA is believed to be decarboxylated by the KS<sup>Y</sup> domain to yield acetyl CoA. The present invention provides recombinant epothilone derivative loading domains or their encoding DNA sequences in which the malonyl specific AT domain or its encoding sequence has been changed to another specificity, such as methylmalonyl CoA, ethylmalonyl CoA, and

25 2-hydroxymalonyl CoA. When expressed with the other proteins of the epothilone PKS, such loading domains lead to the production of epothilones in which the methyl substituent of the thiazole ring of epothilone is replaced with, respectively, ethyl, propyl, and hydroxymethyl. The present invention provides recombinant PKS enzymes comprising such loading domains and host cells for producing such enzymes and the

30 polyketides produced thereby.

Those of skill in the art will recognize that an AT domain that is specific for 2-hydroxymalonyl CoA will result in a polyketide with a hydroxyl group at the corresponding location in the polyketide produced, and that the hydroxyl group can be

methyated to yield a methoxy group by polyketide modification enzymes. See, e.g., the patent applications cited in connection with the FK-520 PKS in the table below.

Consequently, reference to a PKS that has a 2-hydroxymalonyl specific AT domain herein similarly refers to polyketides produced by that PKS that have either a hydroxyl or  
5 methoxyl group at the corresponding location in the polyketide.

The loading domain of the epothilone PKS also comprises an ER domain. While, this ER domain may be involved in forming one of the double bonds in the thiazole moiety in epothilone (in the reverse of its normal reaction), or it may be non-functional. In either event, the invention provides recombinant DNA compounds that encode the  
10 epothilone PKS loading domain with and without the ER region, as well as hybrid loading domains that contain an ER domain from another PKS (either active or inactive, with or without accompanying KR and DH domains) in place of the ER domain of the epothilone loading domain. The present invention also provides recombinant PKS enzymes comprising such loading domains and host cells for producing such enzymes and the  
15 polyketides produced thereby.

The recombinant nucleic acid compounds of the invention that encode the loading domain of the epothilone PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the epothilone loading domain is coexpressed with the proteins of a  
20 heterologous PKS. As used herein, reference to a heterologous modular PKS (or to the coding sequence therefor) refers to all or part of a PKS, including each of the multiple proteins constituting the PKS, that synthesizes a polyketide other than an epothilone or epothilone derivative (or to the coding sequences therefor). This coexpression can be in one of two forms. The epothilone loading domain can be coexpressed as a discrete protein  
25 with the other proteins of the heterologous PKS or as a fusion protein in which the loading domain is fused to one or more modules of the heterologous PKS. In either event, the hybrid PKS formed, in which the loading domain of the heterologous PKS is replaced by the epothilone loading domain, provides a novel PKS. Examples of a heterologous PKS that can be used to prepare such hybrid PKS enzymes of the invention include but are not  
30 limited to DEBS and the picromycin (narbonolide), oleandolide, rapamycin, FK-506, FK-520, rifamycin, and avermectin PKS enzymes and their corresponding coding sequences.

In another embodiment, a nucleic acid compound comprising a sequence that encodes the epothilone loading domain is coexpressed with the proteins that constitute the

remainder of the epothilone PKS (i.e., the *epoB*, *epoC*, *epoD*, *epoE*, and *epoF* gene products) or a recombinant epothilone PKS that produces an epothilone derivative due to an alteration or mutation in one or more of the *epoB*, *epoC*, *epoD*, *epoE*, and *epoF* genes. As used herein, reference to an epothilone or a PKS that produces an epothilone derivative (or to the coding sequence therefor) refers to all or any one of the proteins that comprise the PKS (or to the coding sequences therefor).

In another embodiment, the invention provides recombinant nucleic acid compounds that encode a loading domain composed of part of the epothilone loading domain and part of a heterologous PKS. In this embodiment, the invention provides, for example, either replacing the malonyl CoA specific AT with a methylmalonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT. This replacement, like the others described herein, is typically mediated by replacing the coding sequences therefor to provide a recombinant DNA compound of the invention; the recombinant DNA is used to prepare the corresponding protein. Such changes (including not only replacements but also deletions and insertions) may be referred to herein either at the DNA or protein level.

The compounds of the invention also include those in which both the KS<sup>Y</sup> and AT domains of the epothilone loading domain have been replaced but the ACP and/or linker regions of the epothilone loading domain are left intact. Linker regions are those segments of amino acids between domains in the loading domain and modules of a PKS that help form the tertiary structure of the protein and are involved in correct alignment and positioning of the domains of a PKS. These compounds include, for example, a recombinant loading domain coding sequence in which the KS<sup>Y</sup> and AT domain coding sequences of the epothilone PKS have been replaced by the coding sequences for the KS<sup>Q</sup> and AT domains of, for example, the oleandolide PKS or the narbonolide PKS. There are also PKS enzymes that do not employ a KS<sup>Q</sup> domain but instead merely utilize an AT domain that binds acetyl CoA, propionyl CoA, or butyryl CoA (the DEBS loading domain) or isobutyryl CoA (the avermectin loading domain). Thus, the compounds of the invention also include, for example, a recombinant loading domain coding sequence in which the KS<sup>Y</sup> and AT domain coding sequences of the epothilone PKS have been replaced by an AT domain of the DEBS or avermectin PKS. The present invention also provides recombinant DNA compounds encoding loading domains in which the ACP domain or any of the linker regions of the epothilone loading domain has been replaced by another ACP or linker region.

Any of the above loading domain coding sequences is coexpressed with the other proteins that constitute a PKS that synthesizes epothilone, an epothilone derivative, or another polyketide to provide a PKS of the invention. If the product desired is epothilone or an epothilone derivative, then the loading domain coding sequence is typically  
5 expressed as a discrete protein, as is the loading domain in the naturally occurring epothilone PKS. If the product desired is produced by the loading domain of the invention and proteins from one or more non-epothilone PKS enzymes, then the loading domain is expressed either as a discrete protein or as a fusion protein with one or more modules of the heterologous PKS.

10 The present invention also provides hybrid PKS enzymes in which the epothilone loading domain has been replaced in its entirety by a loading domain from a heterologous PKS with the remainder of the PKS proteins provided by modified or unmodified epothilone PKS proteins. The present invention also provides recombinant expression vectors and host cells for producing such enzymes and the polyketides produced thereby.  
15 In one embodiment, the heterologous loading domain is expressed as a discrete protein in a host cell that expresses the *epoB*, *epoC*, *epoD*, *epoE*, and *epoF* gene products. In another embodiment, the heterologous loading domain is expressed as a fusion protein with the *epoB* gene product in a host cell that expresses the *epoC*, *epoD*, *epoE*, and *epoF* gene products. In a related embodiment, the present invention provides recombinant epothilone  
20 PKS enzymes in which the loading domain has been deleted and replaced by an NRPS module and corresponding recombinant DNA compounds and expression vectors. In this embodiment, the recombinant PKS enzymes thus produce an epothilone derivative that comprises a dipeptide moiety, as in the compound leinamycin. The invention provides such enzymes in which the remainder of the epothilone PKS is identical in function to the  
25 native epothilone PKS as well as those in which the remainder is a recombinant PKS that produces an epothilone derivative of the invention.

The present invention also provides reagents and methods useful in deleting the loading domain coding sequence or any portion thereof from the chromosome of a host cell, such as *Sorangium cellulosum*, or replacing those sequences or any portion thereof  
30 with sequences encoding a recombinant loading domain. Using a recombinant vector that comprises DNA complementary to the DNA including and/or flanking the loading domain coding sequence in the *Sorangium* chromosome, one can employ the vector and

homologous recombination to replace the native loading domain coding sequence with a recombinant loading domain coding sequence or to delete the sequence altogether.

Moreover, while the above discussion focuses on deleting or replacing the epothilone loading domain coding sequences, those of skill in the art will recognize that the present invention provides recombinant DNA compounds, vectors, and methods useful in deleting or replacing all or any portion of an epothilone PKS gene or an epothilone modification enzyme gene. Such methods and materials are useful for a variety of purposes. One purpose is to construct a host cell that does not make a naturally occurring epothilone or epothilone derivative. For example, a host cell that has been modified to not produce a naturally occurring epothilone may be particularly preferred for making epothilone derivatives or other polyketides free of any naturally occurring epothilone. Another purpose is to replace the deleted gene with a gene that has been altered so as to provide a different product or to produce more of one product than another.

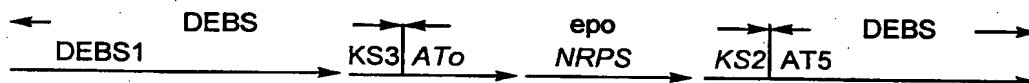
If the epothilone loading domain coding sequence has been deleted or otherwise rendered non-functional in a *Sorangium cellulosum* host cell, then the resulting host cell will produce a non-functional epothilone PKS. This PKS could still bind and process extender units, but the thiazole moiety of epothilone would not form, leading to the production of a novel epothilone derivative. Because this derivative would predictably contain a free amino group, it would be produced at most in low quantities. As noted above, however, provision of a heterologous or other recombinant loading domain to the host cell would result in the production of an epothilone derivative with a structure determined by the loading domain provided.

The loading domain of the epothilone PKS is followed by the first module of the PKS, which is an NRPS module specific for cysteine. This NRPS module is naturally expressed as a discrete protein, the product of the *epoB* gene. The present invention provides the *epoB* gene in recombinant form. The recombinant nucleic acid compounds of the invention that encode the NRPS module of the epothilone PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a nucleic acid compound comprising a sequence that encodes the epothilone NRPS module is coexpressed with genes encoding one or more proteins of a heterologous PKS. The NRPS module can be expressed as a discrete protein or as a fusion protein with one of the proteins of the heterologous PKS. The resulting PKS, in which at least a module of the heterologous PKS is replaced by the epothilone NRPS module or the NRPS module is in

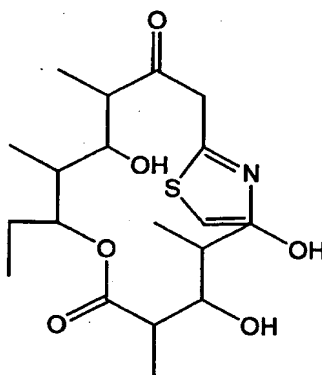
effect added as a module to the heterologous PKS, provides a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the epothilone NRPS module is coexpressed with the other epothilone PKS proteins or modified versions thereof to provide a recombinant epothilone PKS that produces an epothilone or an epothilone derivative.

Two hybrid PKS enzymes provided by the invention illustrate this aspect. Both hybrid PKS enzymes are hybrids of DEBS and the epothilone NRPS module. The first hybrid PKS is composed of four proteins: (i) DEBS1; (ii) a fusion protein composed of the KS domain of module 3 of DEBS and all but the KS domain of the loading domain of the epothilone PKS; (iii) the epothilone NRPS module; and (iv) a fusion protein composed of the KS domain of module 2 of the epothilone PKS fused to the AT domain of module 5 of DEBS and the rest of DEBS3. This hybrid PKS produces a novel polyketide with a thiazole moiety incorporated into the macrolactone ring and a molecular weight of 413.53 when expressed in *Streptomyces coelicolor*. Glycosylated, hydroxylated, and methylated derivatives can be produced by expression of the hybrid PKS in *Saccharopolyspora erythraea*.

Diagrammatically, the construct is represented:



The structure of the product is:

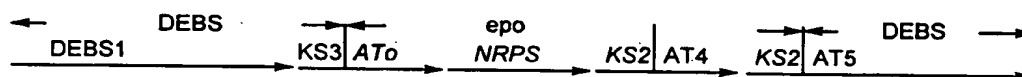


The second hybrid PKS illustrating this aspect of the invention is composed of five proteins: (i) DEBS1; (ii) a fusion protein composed of the KS domain of module 3 of DEBS and all but the KS domain of the loading domain of the epothilone PKS; (iii) the epothilone NRPS module; and (iv) a fusion protein composed of the KS domain of module

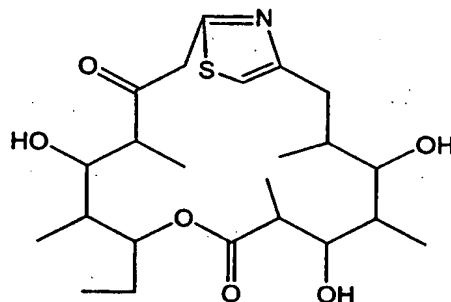


2 of the epothilone PKS fused to the AT domain of module 4 of DEBS and the rest of DEBS2; and (v) DEBS3. This hybrid PKS produces a novel polyketide with a thiazole moiety incorporated into the macrolactone ring and a molecular weight of 455.61 when expressed in *Streptomyces coelicolor*. Glycosylated, hydroxylated, and methylated derivatives can be produced by expression of the hybrid PKS in *Saccharopolyspora erythraea*.

Diagrammatically, the construct is represented:



The structure of the product is:



10

In another embodiment, a portion of the NRPS module coding sequence is utilized in conjunction with a heterologous coding sequence. In this embodiment, the invention provides, for example, changing the specificity of the NRPS module of the epothilone PKS from a cysteine to another amino acid. This change is accomplished by constructing a coding sequence in which all or a portion of the epothilone PKS NRPS module coding sequences have been replaced by those coding for an NRPS module of a different specificity. In one illustrative embodiment, the specificity of the epothilone NRPS module is changed from cysteine to serine or threonine. When the thus modified NRPS module is expressed with the other proteins of the epothilone PKS, the recombinant PKS produces an epothilone derivative in which the thiazole moiety of epothilone (or an epothilone derivative) is changed to an oxazole or 5-methyloxazole moiety, respectively. Alternatively, the present invention provides recombinant PKS enzymes composed of the products of the *epoA*, *epoC*, *epoD*, *epoE*, and *epoF* genes (or modified versions thereof) without an NRPS module or with an NRPS module from a heterologous PKS. The heterologous NRPS module can be expressed as a discrete protein or as a fusion protein with either the *epoA* or *epoC* genes.

25

The invention also provides methods and reagents useful in changing the specificity of a heterologous NRPS module from another amino acid to cysteine. This change is accomplished by constructing a coding sequence in which the sequences that determine the specificity of the heterologous NRPS module have been replaced by those that specify cysteine from the epothilone NRPS module coding sequence. The resulting heterologous NRPS module is typically coexpressed in conjunction with the proteins constituting a heterologous PKS that synthesizes a polyketide other than epothilone or an epothilone derivative, although the heterologous NRPS module can also be used to produce epothilone or an epothilone derivative.

10 In another embodiment, the invention provides recombinant epothilone PKS enzymes and corresponding recombinant nucleic acid compounds and vectors in which the NRPS module has been inactivated or deleted. Such enzymes, compounds, and vectors are constructed generally in accordance with the teaching for deleting or inactivating the epothilone PKS or modification enzyme genes above. Inactive NRPS module proteins and  
15 the coding sequences therefore provided by the invention include those in which the peptidyl carrier protein (PCP) domain has been wholly or partially deleted or otherwise rendered inactive by changing the active site serine (the site for phosphopantetheinylation) to another amino acid, such as alanine, or the adenylation domains have been deleted or otherwise rendered inactive. In one embodiment, both the loading domain and the NRPS  
20 have been deleted or rendered inactive. In any event, the resulting epothilone PKS can then function only if provided a substrate that binds to the KS domain of module 2 (or a subsequent module) of the epothilone PKS or a PKS for an epothilone derivative. In a method provided by the invention, the thus modified cells are then fed activated acylthioesters that are bound by preferably the second, but potentially any subsequent,  
25 module and processed into novel epothilone derivatives.

Thus, in one embodiment, the invention provides *Sorangium* and non-*Sorangium* host cells that express an epothilone PKS (or a PKS that produces an epothilone derivative) with an inactive NRPS. The host cell is fed activated acylthioesters to produce novel epothilone derivatives of the invention. The host cells expressing, or cell free  
30 extracts containing, the PKS can be fed or supplied with N-acylcysteamine thioesters (NACS) of novel precursor molecules to prepare epothilone derivatives. See U.S. patent application Serial No. 60/117,384, filed 27 Jan. 1999, and PCT patent publication No. US99/03986, both of which are incorporated herein by reference, and Example 6, below.

The second (first non-NRPS) module of the epothilone PKS includes a KS, an AT specific for methylmalonyl CoA, a DH, a KR, and an ACP. This module is encoded by a sequence within an ~13.1 kb EcoRI-NsiI restriction fragment of cosmid pKOS35-70.8A3.

5 The recombinant nucleic acid compounds of the invention that encode the second module of the epothilone PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. The second module of the epothilone PKS is produced as a discrete protein by the *epoC* gene. The present invention provides the *epoC* gene in recombinant form. In one embodiment, a DNA compound comprising a sequence that encodes the epothilone second module is coexpressed with the proteins constituting a  
10 heterologous PKS either as a discrete protein or as a fusion protein with one or more modules of the heterologous PKS. The resulting PKS, in which a module of the heterologous PKS is either replaced by the second module of the epothilone PKS or the latter is merely added to the modules of the heterologous PKS, provides a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the second  
15 module of the epothilone PKS is coexpressed with the other proteins constituting the epothilone PKS or a recombinant epothilone PKS that produces an epothilone derivative.

In another embodiment, all or only a portion of the second module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the  
20 methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting either the DH or KR or both; replacing the DH or KR or both with a DH or KR or both that specify a different stereochemistry; and/or inserting an ER. Generally, any reference herein to inserting or replacing a PKS KR, DH, and/or ER domain includes the replacement of the associated KR, DH, or ER domains in  
25 that module, typically with corresponding domains from the module from which the inserted or replacing domain is obtained. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the epothilone PKS, from a gene for a PKS that produces a polyketide  
30 other than epothilone, or from chemical synthesis. The resulting heterologous second module coding sequence can be coexpressed with the other proteins that constitute a PKS that synthesizes epothilone, an epothilone derivative, or another polyketide. Alternatively, one can delete or replace the second module of the epothilone PKS with a module from a

heterologous PKS, which can be expressed as a discrete protein or as a fusion protein fused to either the *epoB* or *epoD* gene product.

Illustrative recombinant PKS genes of the invention include those in which the AT domain encoding sequences for the second module of the epothilone PKS have been altered or replaced to change the AT domain encoded thereby from a methylmalonyl specific AT to a malonyl specific AT. Such malonyl specific AT domain encoding nucleic acids can be isolated, for example and without limitation, from the PKS genes encoding the narbonolide PKS, the rapamycin PKS (i.e., modules 2 and 12), and the FK-520 PKS (i.e., modules 3, 7, and 8). When such a hybrid second module is coexpressed with the other proteins constituting the epothilone PKS, the resulting epothilone derivative produced is a 16-desmethyl epothilone derivative.

In addition, the invention provides DNA compounds and vectors encoding recombinant epothilone PKS enzymes and the corresponding recombinant proteins in which the KS domain of the second (or subsequent) module has been inactivated or deleted. In a preferred embodiment, this inactivation is accomplished by changing the codon for the active site cysteine to an alanine codon. As with the corresponding variants described above for the NRPS module, the resulting recombinant epothilone PKS enzymes are unable to produce an epothilone or epothilone derivative unless supplied a precursor that can be bound and extended by the remaining domains and modules of the recombinant PKS enzyme. Illustrative diketides are described in Example 6, below.

The third module of the epothilone PKS includes a KS, an AT specific for malonyl CoA, a KR, and an ACP. This module is encoded by a sequence within an ~8 kb BglII-NsiI restriction fragment of cosmid pKOS35-70.8A3.

The recombinant DNA compounds of the invention that encode the third module of the epothilone PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. The third module of the epothilone PKS is expressed in a protein, the product of the *epoD* gene, which also contains modules 4, 5, and 6. The present invention provides the *epoD* gene in recombinant form. The present invention also provides recombinant DNA compounds that encode each of the epothilone PKS modules 3, 4, 5, and 6, as discrete coding sequences without coding sequences for the other epothilone modules. In one embodiment, a DNA compound comprising a sequence that encodes the epothilone third module is coexpressed with proteins constituting a heterologous PKS. The third module of the epothilone PKS can be expressed either as a

discrete protein or as a fusion protein fused to one or more modules of the heterologous PKS. The resulting PKS, in which a module of the heterologous PKS is either replaced by that for the third module of the epothilone PKS or the latter is merely added to the modules of the heterologous PKS, provides a novel PKS. In another embodiment, a DNA  
5 compound comprising a sequence that encodes the third module of the epothilone PKS is coexpressed with proteins comprising the remainder of the epothilone PKS or a recombinant epothilone PKS that produces an epothilone derivative, typically as a protein comprising not only the third but also the fourth, fifth, and sixth modules.

In another embodiment, all or a portion of the third module coding sequence is  
10 utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the malonyl CoA specific AT with a methylmalonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting the KR; replacing the KR with a KR that specifies a different stereochemistry; and/or inserting a DH or a DH and an ER. As above, the reference to  
15 inserting a DH or a DH and an ER includes the replacement of the KR with a DH and KR or an ER, DH, and KR. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the epothilone PKS, from a coding sequence for a PKS that produces a  
20 polyketide other than epothilone, or from chemical synthesis. The resulting heterologous third module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes epothilone, an epothilone derivative, or another polyketide.

Illustrative recombinant PKS genes of the invention include those in which the AT domain encoding sequences for the third module of the epothilone PKS have been altered  
25 or replaced to change the AT domain encoded thereby from a malonyl specific AT to a methylmalonyl specific AT. Such methylmalonyl specific AT domain encoding nucleic acids can be isolated, for example and without limitation, from the PKS genes encoding DEBS, the narbonolide PKS, the rapamycin PKS, and the FK-520 PKS. When coexpressed with the remaining modules and proteins of the epothilone PKS or an  
30 epothilone PKS derivative, the recombinant PKS produces the 14-methyl epothilone derivatives of the invention.

Those of skill in the art will recognize that the KR domain of the third module of the PKS is responsible for forming the hydroxyl group involved in cyclization of

epothilone. Consequently, abolishing the KR domain of the third module or adding a DH or DH and ER domains will interfere with the cyclization, leading either to a linear molecule or to a molecule cyclized at a different location than is epothilone.

5 The fourth module of the epothilone PKS includes a KS, an AT that can bind either malonyl CoA or methylmalonyl CoA, a KR, and an ACP. This module is encoded by a sequence within an ~10 kb NsiI-HindIII restriction fragment of cosmid pKOS35-70.1A2.

10 The recombinant DNA compounds of the invention that encode the fourth module of the epothilone PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the epothilone fourth module is inserted into a DNA compound that comprises the coding sequence for one or more modules of a heterologous PKS. The resulting construct encodes a protein in which a module of the heterologous PKS is either replaced by that for the fourth module of the epothilone PKS or the latter is merely added to the modules of the heterologous PKS. Together with other proteins that constitute the  
15 heterologous PKS, this protein provides a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the fourth module of the epothilone PKS is expressed in a host cell that also expresses the remaining modules and proteins of the epothilone PKS or a recombinant epothilone PKS that produces an epothilone derivative. For making epothilone or epothilone derivatives, the recombinant fourth module is usually  
20 expressed in a protein that also contains the epothilone third, fifth, and sixth modules or modified versions thereof.

In another embodiment, all or a portion of the fourth module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the malonyl CoA and  
25 methylmalonyl specific AT with a malonyl CoA, methylmalonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting the KR; and/or replacing the KR, including, optionally, to specify a different stereochemistry; and/or inserting a DH or a DH and ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP  
30 coding sequence can originate from a coding sequence for another module of the epothilone PKS, from a gene for a PKS that produces a polyketide other than epothilone, or from chemical synthesis. The resulting heterologous fourth module coding sequence is incorporated into a protein subunit of a recombinant PKS that synthesizes epothilone, an

epothilone derivative, or another polyketide. If the desired polyketide is an epothilone or epothilone derivative, the recombinant fourth module is typically expressed as a protein that also contains the third, fifth, and sixth modules of the epothilone PKS or modified versions thereof. Alternatively, the invention provides recombinant PKS enzymes for  
5 epothilones and epothilone derivatives in which the entire fourth module has been deleted or replaced by a module from a heterologous PKS.

In a preferred embodiment, the invention provides recombinant DNA compounds comprising the coding sequence for the fourth module of the epothilone PKS modified to encode an AT that binds methylmalonyl CoA and not malonyl CoA. These recombinant  
10 molecules are used to express a protein that is a recombinant derivative of the *epoD* protein that comprises the modified fourth module as well as modules 3, 5, and 6, any one or more of which can optionally be in derivative form, of the epothilone PKS. In another preferred embodiment, the invention provides recombinant DNA compounds comprising the coding sequence for the fourth module of the epothilone PKS modified to encode an  
15 AT that binds malonyl CoA and not methylmalonyl CoA. These recombinant molecules are used to express a protein that is a recombinant derivative of the *epoD* protein that comprises the modified fourth module as well as modules 3, 5, and 6, any one or more of which can optionally be in derivative form, of the epothilone PKS.

Prior to the present invention, it was known that *Sorangium cellulosum* produced  
20 epothilones A, B, C, D, E, and F and that epothilones A, C, and E had a hydrogen at C-12, while epothilones B, D, and F had a methyl group at this position. Unappreciated prior to the present invention was the order in which these compounds were synthesized in *S. cellulosum*, and the mechanism by which some of the compounds had a hydrogen at C-12 where others had a methyl group at this position. The present disclosure reveals that  
25 epothilones A and B are derived from epothilones C and D by action of the *epoK* gene product and that the presence of a hydrogen or methyl moiety at C-12 is due to the AT domain of module 4 of the epothilone PKS. This domain can bind either malonyl or methylmalonyl CoA and, consistent with its having greater similarity to malonyl specific AT domains than to methylmalonyl specific AT domains, binds malonyl CoA more often  
30 than methylmalonyl CoA.

Thus, the invention provides recombinant DNA compounds and expression vectors and the corresponding recombinant PKS in which the hybrid fourth module with a methylmalonyl specific AT has been incorporated. The methylmalonyl specific AT coding

sequence can originate, for example and without limitation, from coding sequences for the oleandolide PKS, DEBS, the narbonolide PKS, the rapamycin PKS, or any other PKS that comprises a methylmalonyl specific AT domain. In accordance with the invention, the hybrid fourth module expressed from this coding sequence is incorporated into the

5 epothilone PKS (or the PKS for an epothilone derivative), typically as a derivative *epoD* gene product. The resulting recombinant epothilone PKS produces epothilones with a methyl moiety at C-12, i.e., epothilone H (or an epothilone H derivative) if there is no dehydratase activity to form the C-12-C-13 alkene; epothilone D (or an epothilone D derivative), if the dehydratase activity but not the epoxidase activity is present; epothilone

10 B (or an epothilone B derivative), if both the dehydratase and epoxidase activity but not the hydroxylase activity are present; and epothilone F (or an epothilone F derivative), if all three dehydratase, epoxidase, and hydroxylase activities are present. As indicated parenthetically above, the cell will produce the corresponding epothilone derivative if there have been other changes to the epothilone PKS.

15 If the recombinant PKS comprising the hybrid methylmalonyl specific fourth module is expressed in, for example, *Sorangium cellulosum*, the appropriate modifying enzymes are present (unless they have been rendered inactive in accordance with the methods herein), and epothilones D, B, and/or F are produced. Such production is typically carried out in a recombinant *S. cellulosum* provided by the present invention in

20 which the native epothilone PKS is unable to function at all or unable to function except in conjunction with the recombinant fourth module provided. In an illustrative example, one can use the methods and reagents of the invention to render inactive the *epoD* gene in the native host. Then, one can transform that host with a vector comprising the recombinant *epoD* gene containing the hybrid fourth module coding sequence. The recombinant vector

25 can exist as an extrachromosomal element or as a segment of DNA integrated into the host cell chromosome. In the latter embodiment, the invention provides that one can simply integrate the recombinant methylmalonyl specific module 4 coding sequence into wild-type *S. cellulosum* by homologous recombination with the native *epoD* gene to ensure that only the desired epothilone is produced. The invention provides that the *S. cellulosum* host

30 can either express or not express (by mutation or homologous recombination of the native genes therefor) the dehydratase, epoxidase, and/or oxidase gene products and thus form or not form the corresponding epothilone D, B, and F compounds, as the practitioner elects.



*Sorangium cellulosum* modified as described above is only one of the recombinant host cells provided by the invention. In a preferred embodiment, the recombinant methylmalonyl specific epothilone fourth module coding sequences are used in accordance with the methods of invention to produce epothilone D, B, and F (or their  
5 corresponding derivatives) in heterologous host cells. Thus, the invention provides reagents and methods for introducing the epothilone or epothilone derivative PKS and epothilone dehydratase, epoxidase, and hydroxylase genes and combinations thereof into heterologous host cells.

The recombinant methylmalonyl specific epothilone fourth module coding  
10 sequences provided by the invention afford important alternative methods for producing desired epothilone compounds in host cells. Thus, the invention provides a hybrid fourth module coding sequence in which, in addition to the replacement of the endogenous AT coding sequence with a coding sequence for an AT specific for methylmalonyl Co A, coding sequences for a DH and KR for, for example and without limitation, module 10 of  
15 the rapamycin PKS or modules 1 or 5 of the FK-520 PKS have replaced the endogenous KR coding sequences. When the gene product comprising the hybrid fourth module and epothilone PKS modules 3, 5, and 6 (or derivatives thereof) encoded by this coding sequence is incorporated into a PKS comprising the other epothilone PKS proteins (or derivatives thereof) produced in a host cell, the cell makes either epothilone D or its trans  
20 stereoisomer (or derivatives thereof), depending on the stereochemical specificity of the inserted DH and KR domains.

Similarly, and as noted above, the invention provides recombinant DNA compounds comprising the coding sequence for the fourth module of the epothilone PKS modified to encode an AT that binds malonyl CoA and not methylmalonyl CoA. The  
25 invention provides recombinant DNA compounds and vectors and the corresponding recombinant PKS in which this hybrid fourth module has been incorporated into a derivative *epoD* gene product. When incorporated into the epothilone PKS (or the PKS for an epothilone derivative), the resulting recombinant epothilone PKS produces epothilones C, A, and E, depending, again, on whether epothilone modification enzymes are present.  
30 As noted above, depending on the host, whether the fourth module includes a KR and DH domain, and on whether and which of the dehydratase, epoxidase, and oxidase activities are present, the practitioner of the invention can produce one or more of the epothilone G,

C, A, and E compounds and derivatives thereof using the compounds, host cells, and methods of the invention.

The fifth module of the epothilone PKS includes a KS, an AT that binds malonyl CoA, a DH, an ER, a KR, and an ACP. This module is encoded by a sequence within an  
5 ~12.4 kb NsiI-NotI restriction fragment of cosmid pKOS35-70.1A2.

The recombinant DNA compounds of the invention that encode the fifth module of the epothilone PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the epothilone fifth module is inserted into a DNA compound that comprises the  
10 coding sequence for one or more modules of a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the fifth module of the epothilone PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, can be incorporated into an expression vector and used to produce the recombinant protein encoded thereby. When the  
15 recombinant protein is combined with the other proteins of the heterologous PKS, a novel PKS is produced. In another embodiment, a DNA compound comprising a sequence that encodes the fifth module of the epothilone PKS is inserted into a DNA compound that comprises coding sequences for the epothilone PKS or a recombinant epothilone PKS that produces an epothilone derivative. In the latter constructs, the epothilone fifth module is  
20 typically expressed as a protein comprising the third, fourth, and sixth modules of the epothilone PKS or derivatives thereof.

In another embodiment, a portion of the fifth module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module coding sequence and the hybrid module encoded thereby. In this embodiment, the invention provides, for  
25 example, either replacing the malonyl CoA specific AT with a methylmalonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting any one, two, or all three of the ER, DH, and KR; and/or replacing any one, two, or all three of the ER, DH, and KR with either a KR, a DH and KR, or a KR, DH, and ER, including, optionally, to specify a different stereochemistry. In addition, the KS and/or ACP can be replaced with  
30 another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the epothilone PKS, from a coding sequence for a PKS that produces a polyketide other than epothilone, or from chemical synthesis. The resulting hybrid fifth

module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes epothilone, an epothilone derivative, or another polyketide. Alternatively, the fifth module of the epothilone PKS can be deleted or replaced in its entirety by a module of a heterologous PKS to produce a protein that in combination with the other  
5 proteins of the epothilone PKS or derivatives thereof constitutes a PKS that produces an epothilone derivative.

Illustrative recombinant PKS genes of the invention include recombinant *epoD* gene derivatives in which the AT domain encoding sequences for the fifth module of the epothilone PKS have been altered or replaced to change the AT domain encoded thereby  
10 from a malonyl specific AT to a methylmalonyl specific AT. Such methylmalonyl specific AT domain encoding nucleic acids can be isolated, for example and without limitation, from the PKS genes encoding DEBS, the narbonolide PKS, the rapamycin PKS, and the FK-520 PKS. When such recombinant *epoD* gene derivatives are coexpressed with the *epoA*, *epoB*, *epoC*, *epoE*, and *epoF* genes (or derivatives thereof), the PKS composed  
15 thereof produces the 10-methyl epothilones or derivatives thereof. Another recombinant *epoD* gene derivative provided by the invention includes not only this altered module 5 coding sequence but also module 4 coding sequences that encode an AT domain that binds only methylmalonyl CoA. When incorporated into a PKS with the *epoA*, *epoB*, *epoC*, *epoE*, and *epoF* genes, the recombinant *epoD* gene derivative product leads to the  
20 production of 10-methyl epothilone B and/or D derivatives.

Other illustrative recombinant *epoD* gene derivatives of the invention include those in which the ER, DH, and KR domain encoding sequences for the fifth module of the epothilone PKS have been replaced with those encoding (i) a KR and DH domain; (ii) a KR domain; and (iii) an inactive KR domain. These recombinant *epoD* gene derivatives of  
25 the invention are coexpressed with the *epoA*, *epoB*, *epoC*, *epoE*, and *epoF* genes to produce a recombinant PKS that makes the corresponding (i) C-11 alkene, (ii) C-11 hydroxy, and (iii) C-11 keto epothilone derivatives. These recombinant *epoD* gene derivatives can also be coexpressed with recombinant *epo* genes containing other alterations or can themselves be further altered to produce a PKS that makes the  
30 corresponding C-11 epothilone derivatives. For example, one recombinant *epoD* gene derivative provided by the invention also includes module 4 coding sequences that encode an AT domain that binds only methylmalonyl CoA. When incorporated into a PKS with the *epoA*, *epoB*, *epoC*, *epoE*, and *epoF* genes, the recombinant *epoD* gene derivative

product leads to the production of the corresponding C-11 epothilone B and/or D derivatives.

Functionally similar *epoD* genes for producing the epothilone C-11 derivatives can also be made by inactivation of one, two, or all three of the ER, DH, and KR domains of the epothilone fifth module. However, the preferred mode for altering such domains in any module is by replacement with the complete set of desired domains taken from another module of the same or a heterologous PKS coding sequence. In this manner, the natural architecture of the PKS is conserved. Also, when present, KR and DH or KR, DH, and ER domains that function together in a native PKS are preferably used in the recombinant PKS. Illustrative replacement domains for the substitutions described above include, for example and without limitation, the inactive KR domain from the rapamycin PKS module 3 to form the ketone, the KR domain from the rapamycin PKS module 5 to form the alcohol, and the KR and DH domains from the rapamycin PKS module 4 to form the alkene. Other such inactive KR, active KR, and active KR and DH domain encoding nucleic acids can be isolated from, for example and without limitation, the PKS genes encoding DEBS, the narbonolide PKS, and the FK-520 PKS. Each of the resulting PKS enzymes produces a polyketide compound that comprises a functional group at the C-11 position that can be further derivatized *in vitro* by standard chemical methodology to yield semi-synthetic epothilone derivatives of the invention.

The sixth module of the epothilone PKS includes a KS, an AT that binds methylmalonyl CoA, a DH, an ER, a KR, and an ACP. This module is encoded by a sequence within an ~14.5 kb HindIII-NsiI restriction fragment of cosmid pKOS35-70.1A2.

The recombinant DNA compounds of the invention that encode the sixth module of the epothilone PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the epothilone sixth module is inserted into a DNA compound that comprises the coding sequence for one or more modules of a heterologous PKS. The resulting protein encoded by the construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the sixth module of the epothilone PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS when coexpressed with the other proteins comprising the PKS. In another embodiment, a DNA compound comprising a sequence that encodes the sixth module of

the epothilone PKS is inserted into a DNA compound that comprises the coding sequence for modules 3, 4, and 5 of the epothilone PKS or a recombinant epothilone PKS that produces an epothilone derivative and coexpressed with the other proteins of the epothilone or epothilone derivative PKS to produce a PKS that makes epothilone or an epothilone derivative in a host cell.

In another embodiment, a portion of the sixth module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting any one, two, or all three of the ER, DH, and KR; and/or replacing any one, two, or all three of the ER, DH, and KR with either a KR, a DH and KR, or a KR, DH, and ER, including, optionally, to specify a different stereochemistry. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the epothilone PKS, from a coding sequence for a PKS that produces a polyketide other than epothilone, or from chemical synthesis. The resulting heterologous sixth module coding sequence can be utilized in conjunction with a coding sequence for a protein subunit of a PKS that makes epothilone, an epothilone derivative, or another polyketide. If the PKS makes epothilone or an epothilone derivative, the hybrid sixth module is typically expressed as a protein comprising modules 3, 4, and 5 of the epothilone PKS or derivatives thereof. Alternatively, the sixth module of the epothilone PKS can be deleted or replaced in its entirety by a module from a heterologous PKS to produce a PKS for an epothilone derivative.

Illustrative recombinant PKS genes of the invention include those in which the AT domain encoding sequences for the sixth module of the epothilone PKS have been altered or replaced to change the AT domain encoded thereby from a methylmalonyl specific AT to a malonyl specific AT. Such malonyl specific AT domain encoding nucleic acids can be isolated from, for example and without limitation, the PKS genes encoding the narbonolide PKS, the rapamycin PKS, and the FK-520 PKS. When a recombinant *epoD* gene of the invention encoding such a hybrid module 6 is coexpressed with the other epothilone PKS genes, the recombinant PKS makes the 8-desmethyl epothilone derivatives. This recombinant *epoD* gene derivative can also be coexpressed with

recombinant *epo* gene derivatives containing other alterations or can itself be further altered to produce a PKS that makes the corresponding 8-desmethyl epothilone derivatives. For example, one recombinant *epoD* gene provided by the invention also includes module 4 coding sequences that encode an AT domain that binds only methylmalonyl CoA. When incorporated into a PKS with the *epoA*, *epoB*, *epoC*, *epoE*, and *epoF* genes, the recombinant *epoD* gene product leads to the production of the 8-desmethyl derivatives of epothilones B and D.

Other illustrative recombinant *epoD* gene derivatives of the invention include those in which the ER, DH, and KR domain encoding sequences for the sixth module of the epothilone PKS have been replaced with those that encode (i) a KR and DH domain; (ii) a KR domain; and (iii) an inactive KR domain. These recombinant *epoD* gene derivatives of the invention, when coexpressed with the other epothilone PKS genes make the corresponding (i) C-9 alkene, (ii) C-9 hydroxy, and (iii) C-9 keto epothilone derivatives. These recombinant *epoD* gene derivatives can also be coexpressed with other recombinant *epo* gene derivatives containing other alterations or can themselves be further altered to produce a PKS that makes the corresponding C-9 epothilone derivatives. For example, one recombinant *epoD* gene derivative provided by the invention also includes module 4 coding sequences that encode an AT domain that binds only methylmalonyl CoA. When incorporated into a PKS with the *epoA*, *epoB*, *epoC*, *epoE*, and *epoF* genes, the recombinant *epoD* gene product leads to the production of the C-9 derivatives of epothilones B and D.

Functionally equivalent sixth modules can also be made by inactivation of one, two, or all three of the ER, DH, and KR domains of the epothilone sixth module. The preferred mode for altering such domains in any module is by replacement with the complete set of desired domains taken from another module of the same or a heterologous PKS coding sequence. Illustrative replacement domains for the substitutions described above include but are not limited to the inactive KR domain from the rapamycin PKS module 3 to form the ketone, the KR domain from the rapamycin PKS module 5 to form the alcohol, and the KR and DH domains from the rapamycin PKS module 4 to form the alkene. Other such inactive KR, active KR, and active KR and DH domain encoding nucleic acids can be isolated from for example and without limitation the PKS genes encoding DEBS, the narbonolide PKS, and the FK-520 PKS. Each of the resulting PKSs produces a polyketide compound that comprises a functional group at the C-9 position that

can be further derivatized *in vitro* by standard chemical methodology to yield semi-synthetic epothilone derivatives of the invention.

The seventh module of the epothilone PKS includes a KS, an AT specific for methylmalonyl CoA, a KR, and an ACP. This module is encoded by a sequence within an  
5 ~8.7 kb BglII restriction fragment from cosmid pKOS35-70.4.

The recombinant DNA compounds of the invention that encode the seventh module of the epothilone PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. The seventh module of the epothilone PKS is contained in the gene product of the *epoE* gene, which also contains the eighth module.  
10 The present invention provides the *epoE* gene in recombinant form, but also provides DNA compounds that encode the seventh module without coding sequences for the eighth module as well as DNA compounds that encode the eighth module without coding sequences for the seventh module. In one embodiment, a DNA compound comprising a sequence that encodes the epothilone seventh module is inserted into a DNA compound  
15 that comprises the coding sequence for one or more modules of a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the seventh module of the epothilone PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence that can be expressed in a host cell. Alternatively, the epothilone  
20 seventh module can be expressed as a discrete protein. In another embodiment, a DNA compound comprising a sequence that encodes the seventh module of the epothilone PKS is expressed to form a protein that, together with other proteins, constitutes the epothilone PKS or a PKS that produces an epothilone derivative. In these embodiments, the seventh module is typically expressed as a protein comprising the eighth module of the epothilone  
25 PKS or a derivative thereof and coexpressed with the *epoA*, *epoB*, *epoC*, *epoD*, and *epoF* genes or derivatives thereof to constitute the PKS.

In another embodiment, a portion or all of the seventh module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA  
30 specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting the KR; replacing the KR with a KR that specifies a different stereochemistry; and/or inserting a DH or a DH and an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or

insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the epothilone PKS, from a coding sequence for a PKS that produces a polyketide other than epothilone, or from chemical synthesis. The resulting heterologous seventh module coding sequence is utilized, optionally in conjunction with other coding sequences, to express a protein that together with other proteins constitutes a PKS that synthesizes epothilone, an epothilone derivative, or another polyketide. When used to prepare epothilone or an epothilone derivative, the seventh module is typically expressed as a protein comprising the eighth module or derivative thereof and coexpressed with the *epoA*, *epoB*, *epoC*, *epoD*, and *epoF* genes or derivatives thereof to constitute the PKS. Alternatively, the coding sequences for the seventh module in the *epoE* gene can be deleted or replaced by those for a heterologous module to prepare a recombinant *epoE* gene derivative that, together with the *epoA*, *epoB*, *epoC*, *epoD*, and *epoF* genes, can be expressed to make a PKS for an epothilone derivative.

Illustrative recombinant *epoE* gene derivatives of the invention include those in which the AT domain encoding sequences for the seventh module of the epothilone PKS have been altered or replaced to change the AT domain encoded thereby from a methylmalonyl specific AT to a malonyl specific AT. Such malonyl specific AT domain encoding nucleic acids can be isolated from for example and without limitation the PKS genes encoding the narbonolide PKS, the rapamycin PKS, and the FK-520 PKS. When coexpressed with the other epothilone PKS genes, *epoA*, *epoB*, *epoC*, *epoD*, and *epoF*, or derivatives thereof, a PKS for an epothilone derivative with a C-6 hydrogen, instead of a C-6 methyl, is produced. Thus, if the genes contain no other alterations, the compounds produced are the 6-desmethyl epothilones.

The eighth module of the epothilone PKS includes a KS, an AT specific for methylmalonyl CoA, inactive KR and DH domains, a methyltransferase (MT) domain, and an ACP. This module is encoded by a sequence within an ~10 kb NotI restriction fragment of cosmid pKOS35-79.85.

The recombinant DNA compounds of the invention that encode the eighth module of the epothilone PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the epothilone eighth module is inserted into a DNA compound that comprises the coding sequence for one or more modules of a heterologous PKS. The



resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the eighth module of the epothilone PKS or the latter is merely added to coding sequences for modules of the heterologous PKS, provides a novel PKS coding sequence that is expressed with the other proteins constituting the PKS to provide a novel PKS. Alternatively, the eighth module can be expressed as a discrete protein that can associate with other PKS proteins to constitute a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the eighth module of the epothilone PKS is coexpressed with the other proteins constituting the epothilone PKS or a PKS that produces an epothilone derivative. In these embodiments, the eighth module is typically expressed as a protein that also comprises the seventh module or a derivative thereof.

In another embodiment, a portion or all of the eighth module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting the inactive KR and/or the inactive DH; replacing the inactive KR and/or DH with an active KR and/or DH; and/or inserting an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the epothilone PKS, from a coding sequence for a PKS that produces a polyketide other than epothilone, or from chemical synthesis. The resulting heterologous eighth module coding sequence is expressed as a protein that is utilized in conjunction with the other proteins that constitute a PKS that synthesizes epothilone, an epothilone derivative, or another polyketide. When used to prepare epothilone or an epothilone derivative, the heterologous or hybrid eighth module is typically expressed as a recombinant *epoE* gene product that also contains the seventh module. Alternatively, the coding sequences for the eighth module in the *epoE* gene can be deleted or replaced by those for a heterologous module to prepare a recombinant *epoE* gene that, together with the *epoA*, *epoB*, *epoC*, *epoD*, and *epoF* genes, can be expressed to make a PKS for an epothilone derivative.

The eighth module of the epothilone PKS also comprises a methylation or methyltransferase (MT) domain with an activity that methylates the epothilone precursor. This function can be deleted to produce a recombinant *epoD* gene derivative of the invention, which can be expressed with the other epothilone PKS genes or derivatives

thereof that makes an epothilone derivative that lacks one or both methyl groups, depending on whether the AT domain of the eighth module has been changed to a malonyl specific AT domain, at the corresponding C-4 position of the epothilone molecule. In another important embodiment, the present invention provides recombinant DNA  
5 compounds that encode a polypeptide with this methylation domain and activity and a variety of recombinant PKS coding sequences that encode recombinant PKS enzymes that incorporate this polypeptide. The availability of this MT domain and the coding sequences therefor provides a significant number of new polyketides that differ from known polyketides by the presence of at least an additional methyl group. The MT domain of the  
10 invention can in effect be added to any PKS module to direct the methylation at the corresponding location in the polyketide produced by the PKS. As but one illustrative example, the present invention provides the recombinant nucleic acid compounds resulting from inserting the coding sequence for this MT activity into a coding sequence for any one or more of the six modules of the DEBS enzyme to produce a recombinant DEBS that  
15 synthesizes a 6-deoxyerythronolide B derivative that comprises one or more additional methyl groups at the C-2, C-4, C-6, C-8, C-10, and/or C-12 positions. In such constructs, the MT domain can be inserted adjacent to the AT or the ACP.

The ninth module of the epothilone PKS includes a KS, an AT specific for malonyl CoA, a KR, an inactive DH, and an ACP. This module is encoded by a sequence within an  
20 ~14.7 HindIII-BglII kb restriction fragment of cosmid pKOS35-79.85.

The recombinant DNA compounds of the invention that encode the ninth module of the epothilone PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. The ninth module of the epothilone PKS is expressed as a protein, the product of the *epoF* gene, that also contains the TE domain of the epothilone  
25 PKS. The present invention provides the *epoF* gene in recombinant form, as well as DNA compounds that encode the ninth module without the coding sequences for the TE domain and DNA compounds that encode the TE domain without the coding sequences for the ninth module. In one embodiment, a DNA compound comprising a sequence that encodes the epothilone ninth module is inserted into a DNA compound that comprises the coding  
30 sequence for one or more modules of a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the ninth module of the epothilone PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS protein coding

sequence that when coexpressed with the other proteins constituting a PKS provides a novel PKS. The ninth module coding sequence can also be expressed as a discrete protein with or without an attached TE domain. In another embodiment, a DNA compound comprising a sequence that encodes the ninth module of the epothilone PKS is expressed  
5 as a protein together with other proteins to constitute an epothilone PKS or a PKS that produces an epothilone derivative. In these embodiments, the ninth module is typically expressed as a protein that also contains the TE domain of either the epothilone PKS or a heterologous PKS.

In another embodiment, a portion or all of the ninth module coding sequence is  
10 utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the malonyl CoA specific AT with a methylmalonyl CoA, ethylmalonyl CoA, or 2-hydroxy malonyl CoA specific AT; deleting the KR; replacing the KR with a KR that specifies a different stereochemistry; and/or inserting a DH or a DH and an ER. In addition, the KS and/or  
15 ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the epothilone PKS, from a coding sequence for a PKS that produces a polyketide other than epothilone, or from chemical synthesis. The resulting heterologous ninth module coding sequence is coexpressed with  
20 the other proteins constituting a PKS that synthesizes epothilone, an epothilone derivative, or another polyketide. Alternatively, the present invention provides a PKS for an epothilone or epothilone derivative in which the ninth module has been replaced by a module from a heterologous PKS or has been deleted in its entirety. In the latter embodiment, the TE domain is expressed as a discrete protein or fused to the eighth  
25 module.

The ninth module of the epothilone PKS is followed by a thioesterase domain. This domain is encoded in the ~14.7 kb HindIII-BglII restriction comprising the ninth module coding sequence. The present invention provides recombinant DNA compounds that encode hybrid PKS enzymes in which the ninth module of the epothilone PKS is fused to  
30 a heterologous thioesterase or one or more modules of a heterologous PKS are fused to the epothilone PKS thioesterase. Thus, for example, a thioesterase domain coding sequence from another PKS can be inserted at the end of the ninth module ACP coding sequence in recombinant DNA compounds of the invention. Recombinant DNA compounds encoding

this thioesterase domain are therefore useful in constructing DNA compounds that encode a protein of the epothilone PKS, a PKS that produces an epothilone derivative, and a PKS that produces a polyketide other than epothilone or an epothilone derivative.

In one important embodiment, the present invention thus provides a hybrid PKS  
5 and the corresponding recombinant DNA compounds that encode the proteins constituting those hybrid PKS enzymes. For purposes of the present invention a hybrid PKS is a recombinant PKS that comprises all or part of one or more modules, loading domain, and thioesterase/cyclase domain of a first PKS and all or part of one or more modules, loading domain, and thioesterase/cyclase domain of a second PKS. In one preferred embodiment,  
10 the first PKS is most but not all of the epothilone PKS, and the second PKS is only a portion or all of a non-epothilone PKS. An illustrative example of such a hybrid PKS includes an epothilone PKS in which the natural loading domain has been replaced with a loading domain of another PKS. Another example of such a hybrid PKS is an epothilone PKS in which the AT domain of module four is replaced with an AT domain from a  
15 heterologous PKS that binds only methylmalonyl CoA. In another preferred embodiment, the first PKS is most but not all of a non-epothilone PKS, and the second PKS is only a portion or all of the epothilone PKS. An illustrative example of such a hybrid PKS includes an erythromycin PKS in which an AT specific for methylmalonyl CoA is replaced with an AT from the epothilone PKS specific for malonyl CoA. Another example  
20 is an erythromycin PKS that includes the MT domain of the epothilone PKS.

Those of skill in the art will recognize that all or part of either the first or second PKS in a hybrid PKS of the invention need not be isolated from a naturally occurring source. For example, only a small portion of an AT domain determines its specificity. See  
25 U.S. patent application Serial No. 09/346,860 and PCT patent application No. WO US99/15047, each of which is incorporated herein by reference. The state of the art in DNA synthesis allows the artisan to construct de novo DNA compounds of size sufficient to construct a useful portion of a PKS module or domain. For purposes of the present invention, such synthetic DNA compounds are deemed to be a portion of a PKS.

The following Table lists references describing illustrative PKS genes and  
30 corresponding enzymes that can be utilized in the construction of the recombinant PKSs and the corresponding DNA compounds that encode them of the invention. Also presented are various references describing polyketide tailoring and modification enzymes and

corresponding genes that can be employed to make the recombinant DNA compounds of the present invention.

#### **Avermectin**

5 U.S. Pat. No. 5,252,474 to Merck.

MacNeil *et al.*, 1993, Industrial Microorganisms: Basic and Applied Molecular Genetics, Baltz, Hegeman, & Skatrud, eds. (ASM), pp. 245-256, A Comparison of the Genes Encoding the Polyketide Synthases for Avermectin, Erythromycin, and Nemadectin.

10 MacNeil *et al.*, 1992, Gene 115: 119-125, Complex Organization of the *Streptomyces avermitilis* genes encoding the avermectin polyketide synthase.

Ikeda and Omura, 1997, Chem. Res. 97: 2599-2609, Avermectin biosynthesis.

#### **Candicidin (FR008)**

Hu *et al.*, 1994, Mol. Microbiol. 14: 163-172.

#### 15 **Erythromycin**

PCT Pub. No. 93/13663 to Abbott.

US Pat. No. 5,824,513 to Abbott.

Donadio *et al.*, 1991, Science 252:675-9.

20 Cortes *et al.*, 8 Nov. 1990, Nature 348:176-8, An unusually large multifunctional polypeptide in the erythromycin producing polyketide synthase of *Saccharopolyspora erythraea*.

#### Glycosylation Enzymes

PCT Pat. App. Pub. No. 97/23630 to Abbott.

#### **FK-506**

25 Motamedi *et al.*, 1998, The biosynthetic gene cluster for the macrolactone ring of the immunosuppressant FK-506, Eur. J. Biochem. 256: 528-534.

Motamedi *et al.*, 1997, Structural organization of a multifunctional polyketide synthase involved in the biosynthesis of the macrolide immunosuppressant FK-506, Eur. J. Biochem. 244: 74-80.

#### 30 Methyltransferase

US 5,264,355, issued 23 Nov. 1993, Methylating enzyme from *Streptomyces* MA6858. 31-O-desmethyl-FK-506 methyltransferase.

Motamedi *et al.*, 1996, Characterization of methyltransferase and hydroxylase genes involved in the biosynthesis of the immunosuppressants FK-506 and FK-520, J. Bacteriol. 178: 5243-5248.

**FK-520**

- 5 U.S. patent application Serial No. 09/154,083, filed 16 Sep. 1998.  
U.S. patent application Serial No. 09/410,551, filed 1 Oct. 1999.  
Nielsen *et al.*, 1991, Biochem. 30:5789-96.

**Lovastatin**

U.S. Pat. No. 5,744,350 to Merck.

10 **Narbomycin**

U.S. patent application Serial No. 60/107,093, filed 5 Nov. 1998.

**Nemadectin**

MacNeil *et al.*, 1993, *supra*.

**Niddamycin**

- 15 Kakavas *et al.*, 1997, Identification and characterization of the niddamycin polyketide synthase genes from *Streptomyces caelestis*, J. Bacteriol. 179: 7515-7522.

**Oleandomycin**

- 20 Swan *et al.*, 1994, Characterisation of a *Streptomyces antibioticus* gene encoding a type I polyketide synthase which has an unusual coding sequence, Mol. Gen. Genet. 242: 358-362.

U.S. patent application Serial No. 60/120,254, filed 16 Feb. 1999, Serial No. 09/\_\_\_\_\_, filed 28 Oct. 1999, claiming priority thereto by inventors S. Shah, M. Betlach, R. McDaniel, and L. Tang, attorney docket No. 30063-20029.00.

- 25 Olano *et al.*, 1998, Analysis of a *Streptomyces antibioticus* chromosomal region involved in oleandomycin biosynthesis, which encodes two glycosyltransferases responsible for glycosylation of the macrolactone ring, Mol. Gen. Genet. 259(3): 299-308.

**Picromycin**

- 30 PCT patent application No. WO US99/11814, filed 28 May 1999.  
U.S. patent application Serial No. 09/320,878, filed 27 May 1999.  
U.S. patent application Serial No. 09/141,908, filed 28 Aug. 1998.

Xue *et al.*, 1998, Hydroxylation of macrolactones YC-17 and narbomycin is mediated by the pikC-encoded cytochrome P450 in *Streptomyces venezuelae*, Chemistry & Biology 5(11): 661-667.

5 Xue *et al.*, Oct. 1998, A gene cluster for macrolide antibiotic biosynthesis in *Streptomyces venezuelae*: Architecture of metabolic diversity, Proc. Natl. Acad. Sci. USA 95: 12111 12116.

#### **Platenolide**

EP Pat. App. Pub. No. 791,656 to Lilly.

#### **Pradimicin**

10 PCT Pat. Pub. No. WO 98/11230 to Bristol-Myers Squibb.

#### **Rapamycin**

Schwecke *et al.*, Aug. 1995, The biosynthetic gene cluster for the polyketide rapamycin, Proc. Natl. Acad. Sci. USA 92:7839-7843.

15 Aparicio *et al.*, 1996, Organization of the biosynthetic gene cluster for rapamycin in *Streptomyces hygroscopicus*: analysis of the enzymatic domains in the modular polyketide synthase, Gene 169: 9-16.

#### **Rifamycin**

PCT Pat. Pub. No. WO 98/07868 to Novartis.

20 August *et al.*, 13 Feb. 1998, Biosynthesis of the ansamycin antibiotic rifamycin: deductions from the molecular analysis of the *rif* biosynthetic gene cluster of *Amiclatopsis mediterranei* S669, Chemistry & Biology, 5(2): 69-79.

#### **Sorangium PKS**

U.S. patent application Serial No. 09/144,085, filed 31 Aug. 1998.

#### **Soraphen**

25 U.S. Pat. No. 5,716,849 to Novartis.

Schupp *et al.*, 1995, J. Bacteriology 177: 3673-3679. A *Sorangium cellulosum* (Myxobacterium) Gene Cluster for the Biosynthesis of the Macrolide Antibiotic Soraphen A: Cloning, Characterization, and Homology to Polyketide Synthase Genes from Actinomycetes.

#### **Spiramycin**

30 U.S. Pat. No. 5,098,837 to Lilly.

#### Activator Gene

U.S. Pat. No. 5,514,544 to Lilly.

**Tylosin**

U.S. Pat. No. 5,876,991 to Lilly.

EP Pub. No. 791,655 to Lilly.

Kuhstoss *et al.*, 1996, Gene 183:231-6., Production of a novel polyketide through  
5 the construction of a hybrid polyketide synthase.

**Tailoring enzymes**

Merson-Davies and Cundliffe, 1994, Mol. Microbiol. 13: 349-355. Analysis of five  
tylosin biosynthetic genes from the tylBA region of the *Streptomyces fradiae* genome.

As the above Table illustrates, there are a wide variety of PKS genes that serve as  
10 readily available sources of DNA and sequence information for use in constructing the  
hybrid PKS-encoding DNA compounds of the invention. Methods for constructing hybrid  
PKS-encoding DNA compounds are described without reference to the epothilone PKS in  
U.S. Patent Nos. 5,672,491 and 5,712,146 and U.S. patent application Serial Nos.  
09/073,538, filed 6 May 1998, and 09/141,908, filed 28 Aug 1998, each of which is  
15 incorporated herein by reference. Preferred PKS enzymes and coding sequences for the  
proteins which constitute them for purposes of isolating heterologous PKS domain coding  
sequences for constructing hybrid PKS enzymes of the invention are the soraphen PKS  
and the PKS described as a *Sorangium* PKS in the above table.

To summarize the functions of the genes cloned and sequenced in Example 1:

<u>Gene</u>	<u>Protein</u>	<u>Modules</u>	<u>Domains Present</u>
<i>epoA</i>	EpoA	Load	Ks <sup>y</sup> mAT ER ACP
<i>epoB</i>	EpoB	1	NRPS, condensation, heterocyclization, adenylation, thiolation, PCP
<i>epoC</i>	EpoC	2	KS mmAT DH KR ACP
<i>epoD</i>	EpoD	3	KS mAT KR ACP
		4	KS mAT KR ACP
		5	KS mAT DH ER KR ACP
		6	KS mmAT DH ER KR ACP
<i>epoE</i>	EpoE	7	KS mmAT KR ACP
		8	KS mmAT MT DH* KR* ACP
<i>epoF</i>	EpoF	9	KS mAT KR DH* ACP TE



NRPS – non-ribosomal peptide synthetase; KS – ketosynthase; mAT – malonyl CoA specifying acyltransferase; mmAT – methylmalonyl CoA specifying acyltransferase; DH – dehydratase; ER – enoylreductase; KR – ketoreductase; MT – methyltransferase; TE thioesterase; \* – inactive domain.

- 5           The hybrid PKS-encoding DNA compounds of the invention can be and often are hybrids of more than two PKS genes. Even where only two genes are used, there are often two or more modules in the hybrid gene in which all or part of the module is derived from a second (or third) PKS gene. Illustrative examples of recombinant epothilone derivative PKS genes of the invention, which are identified by listing the specificities of the hybrid
- 10 modules (the other modules having the same specificity as the epothilone PKS), include:
- (a) module 4 with methylmalonyl specific AT (mm AT) and a KR and module 2 with a malonyl specific AT (m AT) and a KR;
  - (b) module 4 with mM AT and a KR and module 3 with mM AT and a KR;
  - (c) module 4 with mM AT and a KR and module 5 with mM AT and a ER, DH,
  - 15 and KR;
  - (d) module 4 with mM AT and a KR and module 5 with mM AT and a DH and KR;
  - (e) module 4 with mM AT and a KR and module 5 with mM AT and a KR;
  - (f) module 4 with mM AT and a KR and module 5 with mM AT and an inactive
  - 20 KR;
  - (g) module 4 with mM AT and a KR and module 6 with m AT and a ER, DH, and KR;
  - (h) module 4 with mM AT and a KR and module 6 with m AT and a DH and KR;
  - (i) module 4 with mM AT and a KR and module 6 with m AT and a KR;
  - 25 (j) module 4 with mM AT and a KR and module 6 with m AT and an inactive KR;
  - (k) module 4 with mM AT and a KR and module 7 with m AT;
  - (l) hybrids (c) through (f), except that module 5 has a m AT;
  - (m) hybrids (g) through (j) except that module 6 has a mM AT; and
  - (n) hybrids (a) through (m) except that module 4 has a m AT.
- 30   The above list is illustrative only and should not be construed as limiting the invention, which includes other recombinant epothilone PKS genes and enzymes with not only two hybrid modules other than those shown but also with three or more hybrid modules.

Those of skill in the art will appreciate that a hybrid PKS of the invention includes but is not limited to a PKS of any of the following types: (i) an epothilone or epothilone derivative PKS that contains a module in which at least one of the domains is from a heterologous module; (ii) an epothilone or epothilone derivative PKS that contains a  
5 module from a heterologous PKS; (iii) an epothilone or epothilone derivative PKS that contains a protein from a heterologous PKS; and (iv) combinations of the foregoing.

While an important embodiment of the present invention relates to hybrid PKS genes, the present invention also provides recombinant epothilone PKS genes in which there is no second PKS gene sequence present but which differ from the epothilone PKS  
10 gene by one or more deletions. The deletions can encompass one or more modules and/or can be limited to a partial deletion within one or more modules. When a deletion encompasses an entire module other than the NRPS module, the resulting epothilone derivative is at least two carbons shorter than the compound produced from the PKS from which the deleted version was derived. The deletion can also encompass the NRPS  
15 module and/or the loading domain, as noted above. When a deletion is within a module, the deletion typically encompasses a KR, DH, or ER domain, or both DH and ER domains, or both KR and DH domains, or all three KR, DH, and ER domains.

The catalytic properties of the domains and modules of the epothilone PKS and of epothilone modification enzymes can also be altered by random or site specific  
20 mutagenesis of the corresponding genes. A wide variety of mutagenizing agents and methods are known in the art and are suitable for this purpose. The technique known as DNA shuffling can also be employed. See, e.g., U.S. Patent Nos. 5,830,721; 5,811,238; and 5,605,793; and references cited therein, each of which is incorporated herein by reference.

25

#### Recombinant Manipulations

To construct a hybrid PKS or epothilone derivative PKS gene of the invention, or simply to express unmodified epothilone biosynthetic genes, one can employ a technique, described in PCT Pub. No. 98/27203 and U.S. patent application Serial Nos. 08/989,332,  
30 filed 11 Dec. 1997, and 60/129,731, filed 16 April 1999, each of which is incorporated herein by reference, in which the various genes of the PKS are divided into two or more, often three, segments, and each segment is placed on a separate expression vector. In this manner, the full complement of genes can be assembled and manipulated more readily for

heterologous expression, and each of the segments of the gene can be altered, and various altered segments can be combined in a single host cell to provide a recombinant PKS of the invention. This technique makes more efficient the construction of large libraries of recombinant PKS genes, vectors for expressing those genes, and host cells comprising  
5 those vectors. In this and other contexts, the genes encoding the desired PKS are not only present on two or more vectors, but also can be ordered or arranged differently than in the native producer organism from which the genes were derived. Various examples of this technique as applied to the epothilone PKS are described in the Examples below. In one embodiment, the *epoA*, *epoB*, *epoC*, and *epoD* genes are present on a first plasmid, and the  
10 *epoE* and *epoF* and optionally either the *epoK* or the *epoK* and *epoL* genes are present on a second (or third) plasmid.

Thus, in one important embodiment, the recombinant nucleic acid compounds of the invention are expression vectors. As used herein, the term "expression vector" refers to any nucleic acid that can be introduced into a host cell or cell-free transcription and  
15 translation medium. An expression vector can be maintained stably or transiently in a cell, whether as part of the chromosomal or other DNA in the cell or in any cellular compartment, such as a replicating vector in the cytoplasm. An expression vector also comprises a gene that serves to produce RNA that is translated into a polypeptide in the cell or cell extract. Thus, the vector typically includes a promoter to enhance gene  
20 expression but alternatively may serve to incorporate the relevant coding sequence under the control of an endogenous promoter. Furthermore, expression vectors may typically contain additional functional elements, such as resistance-conferring genes to act as selectable markers and regulatory genes to enhance promoter activity.

The various components of an expression vector can vary widely, depending on the intended use of the vector. In particular, the components depend on the host cell(s) in  
25 which the vector will be used or is intended to function. Vector components for expression and maintenance of vectors in *E. coli* are widely known and commercially available, as are vector components for other commonly used organisms, such as yeast cells and *Streptomyces* cells.

30 In one embodiment, the vectors of the invention are used to transform *Sorangium* host cells to provide the recombinant *Sorangium* host cells of the invention. U.S. Pat. No. 5,686,295, incorporated herein by reference, describes a method for transforming *Sorangium* host cells, although other methods may also be employed. *Sorangium* is a

convenient host for expressing epothilone derivatives of the invention in which the recombinant PKS that produces such derivatives is expressed from a recombinant vector in which the epothilone PKS gene promoter is positioned to drive expression of the recombinant coding sequence. The epothilone PKS gene promoter is provided in  
5 recombinant form by the present invention and is an important embodiment thereof. The promoter is contained within an ~500 nucleotide sequence between the end of the transposon sequences and the start site of the open reading frame of the *epoA* gene. Optionally, one can include sequences from further upstream of this 500 bp region in the promoter. Those of skill in the art will recognize that, if a *Sorangium* host that produces  
10 epothilone is used as the host cell, the recombinant vector need drive expression of only a portion of the PKS containing the altered sequences. Thus, such a vector may comprise only a single altered epothilone PKS gene, with the remainder of the epothilone PKS polypeptides provided by the genes in the host cell chromosomal DNA. If the host cell naturally produces an epothilone, the epothilone derivative will thus be produced in a  
15 mixture containing the naturally occurring epothilone(s).

Those of skill will also recognize that the recombinant DNA compounds of the invention can be used to construct *Sorangium* host cells in which one or more genes involved in epothilone biosynthesis have been rendered inactive. Thus, the invention provides such *Sorangium* host cells, which may be preferred host cells for expressing  
20 epothilone derivatives of the invention so that complex mixtures of epothilones are avoided. Particularly preferred host cells of this type include those in which one or more of any of the epothilone PKS gene ORFs has been disrupted, and/or those in which any or more of the epothilone modification enzyme genes have been disrupted. Such host cells are typically constructed by a process involving homologous recombination using a vector  
25 that contains DNA homologous to the regions flanking the gene segment to be altered and positioned so that the desired homologous double crossover recombination event desired will occur.

Homologous recombination can thus be used to delete, disrupt, or alter a gene. In a preferred illustrative embodiment, the present invention provides a recombinant  
30 epothilone producing *Sorangium cellulosum* host cell in which the *epoK* gene has been deleted or disrupted by homologous recombination using a recombinant DNA vector of the invention. This host cell, unable to make the *epoK* epoxidase gene product is unable to make epothilones A and B and so is a preferred source of epothilones C and D.

Homologous recombination can also be used to alter the specificity of a PKS module by replacing coding sequences for the module or domain of a module to be altered with those specifying a module or domain of the desired specificity. In another preferred illustrative embodiment, the present invention provides a recombinant epothilone  
5 producing *Sorangium cellulosum* host cell in which the coding sequence for the AT domain of module 4 encoded by the *epoD* gene has been altered by homologous recombination using a recombinant DNA vector of the invention to encode an AT domain that binds only methylmalonyl CoA. This host cell, unable to make epothilones A, C, and E is a preferred source of epothilones B, D, and F. The invention also provides  
10 recombinant *Sorangium* host cells in which both alterations and deletions of epothilone biosynthetic genes have been made. For example, the invention provides recombinant *Sorangium cellulosum* host cells in which both of the foregoing alteration and deletion have been made, producing a host cell that makes only epothilone D.

In similar fashion, those of skill in the art will appreciate the present invention  
15 provides a wide variety of recombinant *Sorangium cellulosum* host cells that make less complex mixtures of the epothilones than do the wild type producing cells as well as those that make one or more epothilone derivatives. Such host cells include those that make only epothilones A, C, and E; those that make only epothilones B, D, and F, those that make only epothilone D; and those that make only epothilone C.

20 In another preferred embodiment, the present invention provides expression vectors and recombinant *Myxococcus*, preferably *M. xanthus*, host cells containing those expression vectors that express a recombinant epothilone PKS or a PKS for an epothilone derivative. Presently, vectors that replicate extrachromosomally in *M. xanthus* are not known. There are, however, a number of phage known to integrate into *M. xanthus*  
25 chromosomal DNA, including Mx8, Mx9, Mx81, and Mx82. The integration and attachment function of these phages can be placed on plasmids to create phage-based expression vectors that integrate into the *M. xanthus* chromosomal DNA. Of these, phage Mx9 and Mx8 are preferred for purposes of the present invention. Plasmid pPLH343, described in Salmi *et al.*, Feb. 1998, Genetic determinants of immunity and integration of  
30 temperate *Myxococcus xanthus* phage Mx8, J. Bact. 180(3): 614-621, is a plasmid that replicates in *E. coli* and comprises the phage Mx8 genes that encode the attachment and integration functions.

The promoter of the epothilone PKS gene functions in *Myxococcus xanthus* host cells. Thus, in one embodiment, the present invention provides a recombinant promoter for use in recombinant host cells derived from the promoter of the *Sorangium cellulosum* epothilone PKS gene. The promoter can be used to drive expression of one or more epothilone PKS genes or another useful gene product in recombinant host cells. The invention also provides an epothilone PKS expression vector in which one or more of the epothilone PKS or epothilone modification enzyme genes are under the control of their own promoter. Another preferred promoter for use in *Myxococcus xanthus* host cells for purposes of expressing a recombinant PKS of the invention is the promoter of the pilA gene of *M. xanthus*. This promoter, as well as two *M. xanthus* strains that express high levels of gene products from genes controlled by the pilA promoter, a pilA deletion strain and a pilS deletion strain, are described in Wu and Kaiser, Dec. 1997, Regulation of expression of the pilA gene in *Myxococcus xanthus*, J. Bact. 179(24):7748-7758, incorporated herein by reference. Optionally, the invention provides recombinant *Myxococcus* host cells comprising both the pilA and pilS deletions. Another preferred promoter is the starvation dependent promoter of the sdck gene.

Selectable markers for use in *Myxococcus xanthus* include kanamycin, tetracycline, chloramphenicol, zeocin, spectinomycin, and streptomycin resistance conferring genes. The recombinant DNA expression vectors of the invention for use in *Myxococcus* typically include such a selectable marker and may further comprise the promoter derived from an epothilone PKS or epothilone modification enzyme gene.

The present invention provides preferred expression vectors for use in preparing the recombinant *Myxococcus xanthus* expression vectors and host cells of the invention. These vectors, designated plasmids pKOS35-82.1 and pKOS35-82.2 (Figure 3), are able to replicate in *E. coli* host cells as well as integrate into the chromosomal DNA of *M. xanthus*. The vectors comprise the Mx8 attachment and integration genes as well as the pilA promoter with restriction enzyme recognition sites placed conveniently downstream. The two vectors differ from one another merely in the orientation of the pilA promoter on the vector and can be readily modified to include the epothilone PKS and modification enzyme genes of the invention. The construction of the vectors is described in Example 2.

Especially preferred *Myxococcus* host cells of the invention are those that produce an epothilone or epothilone derivative or mixtures of epothilones or epothilone derivatives at equal to or greater than 20 mg/L, more preferably at equal to or greater than 200 mg/L,

and most preferably at equal to or greater than 1 g/L. Especially preferred are *M. xanthus* host cells that produce at these levels. *M. xanthus* host cells that can be employed for purposes of the invention include the DZ1 (Campos *et al.*, 1978, J. Mol. Biol. 119: 167-178, incorporated herein by reference), the TA-producing cell line ATCC 31046, DK1219  
5 (Hodgkin and Kaiser, 1979, Mol. Gen. Genet. 171: 177-191, incorporated herein by reference), and the DK1622 cell lines (Kaiser, 1979, Proc. Natl. Acad. Sci. USA 76: 5952-5956, incorporated herein by reference).

In another preferred embodiment, the present invention provides expression vectors and recombinant *Pseudomonas fluorescens* host cells that contain those expression  
10 vectors and express a recombinant PKS of the invention. A plasmid for use in constructing the *P. fluorescens* expression vectors and host cells of the invention is plasmid pRSF1010, which replicates in *E. coli* and *P. fluorescens* host cells (see Scholz *et al.*, 1989, Gene 75:271-8, incorporated herein by reference). Low copy number replicons and vectors can also be used. As noted above, the invention also provides the promoter of the *Sorangium*  
15 *cellulosum* epothilone PKS and epothilone modification enzyme genes in recombinant form. The promoter can be used to drive expression of an epothilone PKS gene or other gene in *P. fluorescens* host cells. Also, the promoter of the soraphen PKS genes can be used in any host cell in which a *Sorangium* promoter functions. Thus, in one embodiment, the present invention provides an epothilone PKS expression vector for use in *P.*  
20 *fluorescens* host cells.

In another preferred embodiment, the expression vectors of the invention are used to construct recombinant *Streptomyces* host cells that express a recombinant PKS of the invention. *Streptomyces* host cells useful in accordance with the invention include *S. coelicolor*, *S. lividans*, *S. venezuelae*, *S. ambofaciens*, *S. fradiae*, and the like. Preferred  
25 *Streptomyces* host cell/vector combinations of the invention include *S. coelicolor* CH999 and *S. lividans* K4-114 and K4-155 host cells, which do not produce actinorhodin, and expression vectors derived from the pRM1 and pRM5 vectors, as described in U.S. Patent No. 5,830,750 and U.S. patent application Serial Nos. 08/828,898, filed 31 Mar. 1997, and 09/181,833, filed 28 Oct. 1998. Especially preferred *Streptomyces* host cells of the  
30 invention are those that produce an epothilone or epothilone derivative or mixtures of epothilones or epothilone derivatives at equal to or greater than 20 mg/L, more preferably at equal to or greater than 200 mg/L, and most preferably at equal to or greater than 1 g/L. Especially preferred are *S. coelicolor* and *S. lividans* host cells that produce at these levels.

Also, species of the closely related genus *Saccharopolyspora* can be used to produce epothilones, including but not limited to *S. erythraea*.

The present invention provides a wide variety of expression vectors for use in *Streptomyces*. For replicating vectors, the origin of replication can be, for example and without limitation, a low copy number replicon and vectors comprising the same, such as SCP2\* (see Hopwood *et al.*, Genetic Manipulation of *Streptomyces*: A Laboratory manual (The John Innes Foundation, Norwich, U.K., 1985); Lydiate *et al.*, 1985, Gene 35: 223-235; and Kieser and Melton, 1988, Gene 65: 83-91, each of which is incorporated herein by reference), SLP1.2 (Thompson *et al.*, 1982, Gene 20: 51-62, incorporated herein by reference), and pSG5(ts) (Muth *et al.*, 1989, Mol. Gen. Genet. 219: 341-348, and Bierman *et al.*, 1992, Gene 116: 43-49, each of which is incorporated herein by reference), or a high copy number replicon and vectors comprising the same, such as pIJ101 and pJV1 (see Katz *et al.*, 1983, J. Gen. Microbiol. 129: 2703-2714; Vara *et al.*, 1989, J. Bacteriol. 171: 5782-5781; and Servin-Gonzalez, 1993, Plasmid 30: 131-140, each of which is incorporated herein by reference). High copy number vectors are generally, however, not preferred for expression of large genes or multiple genes. For non-replicating and integrating vectors and generally for any vector, it is useful to include at least an *E. coli* origin of replication, such as from pUC, p1P, p1I, and pBR. For phage based vectors, the phage phiC31 and its derivative KC515 can be employed (see Hopwood *et al.*, *supra*). Also, plasmid pSET152, plasmid pSAM, plasmids pSE101 and pSE211, all of which integrate site-specifically in the chromosomal DNA of *S. lividans*, can be employed.

Typically, the expression vector will comprise one or more marker genes by which host cells containing the vector can be identified and/or selected. Useful antibiotic resistance conferring genes for use in *Streptomyces* host cells include the *ermE* (confers resistance to erythromycin and lincomycin), *tsr* (confers resistance to thiostrepton), *aadA* (confers resistance to spectinomycin and streptomycin), *aacC4* (confers resistance to apramycin, kanamycin, gentamicin, geneticin (G418), and neomycin), *hyg* (confers resistance to hygromycin), and *vph* (confers resistance to viomycin) resistance conferring genes.

The recombinant PKS gene on the vector will be under the control of a promoter, typically with an attendant ribosome binding site sequence. A preferred promoter is the *actI* promoter and its attendant activator gene *actII-ORF4*, which is provided in the pRM1 and pRM5 expression vectors, *supra*. This promoter is activated in the stationary phase of



growth when secondary metabolites are normally synthesized. Other useful *Streptomyces* promoters include without limitation those from the *ermE* gene and the *melC1* gene, which act constitutively, and the *tipA* gene and the *merA* gene, which can be induced at any growth stage. In addition, the T7 RNA polymerase system has been transferred to

5 *Streptomyces* and can be employed in the vectors and host cells of the invention. In this system, the coding sequence for the T7 RNA polymerase is inserted into a neutral site of the chromosome or in a vector under the control of the inducible *merA* promoter, and the gene of interest is placed under the control of the T7 promoter. As noted above, one or more activator genes can also be employed to enhance the activity of a promoter.

10 Activator genes in addition to the *actII-ORF4* gene discussed above include *dnrI*, *redD*, and *ptpA* genes (see U.S. patent application Serial No. 09/181,833, *supra*), which can be employed with their cognate promoters to drive expression of a recombinant gene of the invention.

The present invention also provides recombinant expression vectors that drive

15 expression of the epothilone PKS and PKS enzymes that produce epothilone or epothilone derivatives in plant cells. Such vectors are constructed in accordance with the teachings in U.S. patent application Serial No. 09/114,083, filed 10 July 1998, and PCT patent publication No. 99/02669, each of which is incorporated herein by reference. Plants and plant cells expressing epothilone are disease resistant and able to resist fungal infection.

20 For improved production of an epothilone or epothilone derivative in any heterologous host cells, including plant, *Myxococcus*, *Pseudomonas*, and *Streptomyces* host cells, one can also transform the cell to express a heterologous phosphopantetheinyl transferase. See U.S. patent application Serial No. 08/728,742, filed 11 Oct. 1996, and PCT patent publication No. 97/13845, both of which are incorporated herein by reference.

25 In addition to providing recombinant expression vectors that encode the epothilone or an epothilone derivative PKS, the present invention also provides, as discussed above, DNA compounds that encode epothilone modification enzyme genes. As discussed above, these gene products convert epothilones C and D to epothilones A and B, and convert epothilones A and B to epothilones E and F. The present invention also provides

30 recombinant expression vectors and host cells transformed with those vectors that express any one or more of those genes and so produce the corresponding epothilone or epothilone derivative. In one aspect, the present invention provides the *epoK* gene in recombinant

form and host cells that express the gene product thereof, which converts epothilones C and D to epothilones A and B, respectively.

In another important embodiment, and as noted above, the present invention provides vectors for disrupting the function of any one or more of the *epoL*, *epoK*, and any  
5 of the ORFs associated with the epothilone PKS gene cluster in *Sorangium* cells. The invention also provides recombinant *Sorangium* host cells lacking (or containing inactivated forms of) any one or more of these genes. These cells can be used to produce the corresponding epothilones and epothilone derivatives that result from the absence of any one or more of these genes.

10 The invention also provides non-*Sorangium* host cells that contain a recombinant epothilone PKS or a PKS for an epothilone derivative but do not contain (or contain non-functional forms of) any epothilone modification enzyme genes. These host cells of the invention are expected produce epothilones G and H in the absence of a dehydratase activity capable of forming the C-12-C-13 alkene of epothilones C and D. This  
15 dehydration reaction is believed to take place in the absence of the *epoL* gene product in *Streptomyces* host cells. The host cells produce epothilones C and D (or the corresponding epothilone C and D derivative) when the dehydratase activity is present and the P450 epoxidase and hydroxylase (that converts epothilones A and B to epothilones E and F, respectively) genes are absent. The host cells also produce epothilones A and B (or the  
20 corresponding epothilone A and B derivatives) when the hydroxylase gene only is absent. Preferred for expression in these host cells is the recombinant epothilone PKS enzymes of the invention that contain the hybrid module 4 with an AT specific for methylmalonyl CoA only, optionally in combination with one or more additional hybrid modules. Also preferred for expression in these host cells is the recombinant epothilone PKS enzymes of  
25 the invention that contain the hybrid module 4 with an AT specific for malonyl CoA only, optionally in combination with one or more additional hybrid modules.

The recombinant host cells of the invention can also include other genes and corresponding gene products that enhance production of a desired epothilone or epothilone derivative. As but one non-limiting example, the epothilone PKS proteins require  
30 phosphopantetheinylation of the ACP domains of the loading domain and modules 2 through 9 as well as of the PCP domain of the NRPS. Phosphopantethein-ylation is mediated by enzymes that are called phosphopantetheinyl transferases (PPTases). To produce functional PKS enzyme in host cells that do not naturally express a PPTase able

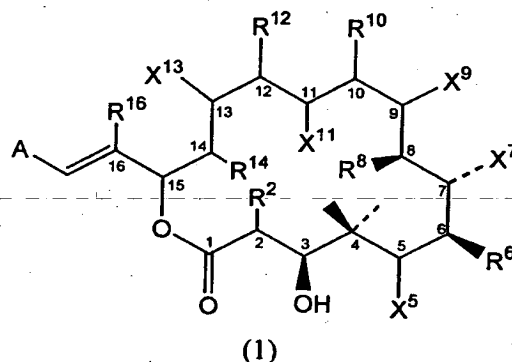
to act on the desired PKS enzyme or to increase amounts of functional PKS enzyme in host cells in which the PPTase is rate-limiting, one can introduce a heterologous PPTase, including but not limited to Sfp, as described in PCT Pat. Pub. Nos. 97/13845 and 98/27203, and U.S. patent application Serial Nos. 08/728,742, filed 11 Oct. 1996, and 5 08/989,332, each of which is incorporated herein by reference.

The host cells of the invention can be grown and fermented under conditions known in the art for other purposes to produce the compounds of the invention. The compounds of the invention can be isolated from the fermentation broths of these cultured cells and purified by standard procedures. Fermentation conditions for producing the 10 compounds of the invention from *Sorangium* host cells can be based on the protocols described in PCT patent publication Nos. 93/10121, 97/19086, 98/22461, and 99/42602, each of which is incorporated herein by reference. The novel epothilone analogs of the present invention, as well as the epothilones produced by the host cells of the invention, can be derivatized and formulated as described in PCT patent publication Nos. 93/10121, 15 97/19086, 98/08849, 98/22461, 98/25929, 99/01124, 99/02514, 99/07692, 99/27890, 99/39694, 99/40047, 99/42602, 99/43653, 99/43320, 99/54319, 99/54319, and 99/54330, and U.S. Patent No. 5,969,145, each of which is incorporated herein by reference.

#### Invention Compounds

20 Preferred compounds of the invention include the 14-methyl epothilone derivatives (made by utilization of the hybrid module 3 of the invention that has an AT that binds methylmalonyl CoA instead of malonyl CoA); the 8,9-dehydro epothilone derivatives (made by utilization of the hybrid module 6 of the invention that has a DH and KR instead of an ER, DH, and KR); the 10-methyl epothilone derivatives (made by utilization of the 25 hybrid module 5 of the invention that has an AT that binds methylmalonyl CoA instead of malonyl CoA); the 9-hydroxy epothilone derivatives (made by utilization of the hybrid module 6 of the invention that has a KR instead of an ER, DH, and KR); the 8-desmethyl-14-methyl epothilone derivatives (made by utilization of the hybrid module 3 of the invention that has an AT that binds methylmalonyl CoA instead of malonyl CoA and a 30 hybrid module 6 that binds malonyl CoA instead of methylmalonyl CoA ); and the 8-desmethyl-8,9-dehydro epothilone derivatives (made by utilization of the hybrid module 6 of the invention that has a DH and KR instead of an ER, DH, and KR and an AT that specifies malonyl CoA instead of methylmalonyl CoA).

More generally, preferred epothilone derivative compounds of the invention are those that can be produced by altering the epothilone PKS genes as described herein and optionally by action of epothilone modification enzymes and/or by chemically modifying the resulting epothilones produced when those genes are expressed. Thus, the present  
 5 invention provides compounds of the formula:



including the glycosylated forms thereof and stereoisomeric forms where the stereochemistry is not shown,

10 wherein A is a substituted or unsubstituted straight, branched chain or cyclic alkyl, alkenyl or alkynyl residue optionally containing 1-3 heteroatoms selected from O, S and N; or wherein A comprises a substituted or unsubstituted aromatic residue;

$R^2$  represents H, H, or H, lower alkyl, or lower alkyl, lower alkyl;

$X^5$  represents =O or a derivative thereof, or H, OH or H, NR<sub>2</sub> wherein R is H, or  
 15 alkyl, or acyl or H, OCOR or H, OCONR<sub>2</sub> wherein R is H or alkyl, or is H, H;

$R^6$  represents H or lower alkyl, and the remaining substituent on the corresponding carbon is H;

$X^7$  represents OR, NR<sub>2</sub>, wherein R is H, or alkyl or acyl or is OCOR, or OCONR<sub>2</sub> wherein R is H or alkyl or  $X^7$  taken together with  $X^9$  forms a carbonate or carbamate  
 20 cycle, and wherein the remaining substituent on the corresponding carbon is H;

$R^8$  represents H or lower alkyl and the remaining substituent on the carbon is H;

$X^9$  represents =O or a derivative thereof, or is H, OR or H, NR<sub>2</sub>, wherein R is H, or alkyl or acyl or is H, OCOR or H, OCONR<sub>2</sub> wherein R is H or alkyl, or represents H, H or wherein  $X^9$  together with  $X^7$  or with  $X^{11}$  can form a cyclic carbonate or carbamate;

25  $R^{10}$  is H, H or H, lower alkyl, or lower alkyl, lower alkyl;

$X^{11}$  is =O or a derivative thereof, or is H, OR, or H, NR<sub>2</sub> wherein R is H, or alkyl or acyl or is H, OCOR or H, OCONR<sub>2</sub> wherein R is H or alkyl, or is H, H or wherein  $X^{11}$  in combination with  $X^9$  may form a cyclic carbonate or carbamate;

$R^{12}$  is H, H, or H, lower alkyl, or lower alkyl, lower alkyl;

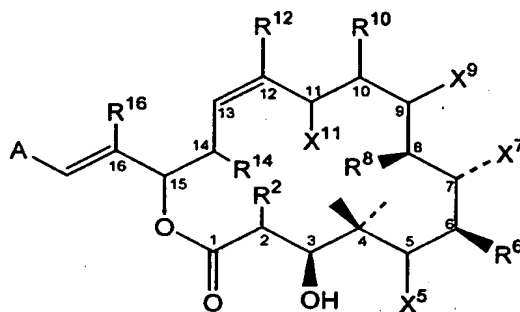
5  $X^{13}$  is =O or a derivative thereof, or H, OR or H, NR<sub>2</sub> wherein R is H, alkyl or acyl or is H, OCOR or H, OCONR<sub>2</sub> wherein R is H or alkyl;

$R^{14}$  is H, H, or H, lower alkyl, or lower alkyl, lower alkyl;

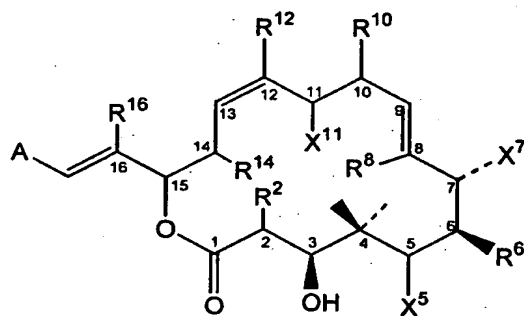
$R^{16}$  is H or lower alkyl; and

wherein optionally H or another substituent may be removed from positions 12 and  
10 13 and/or 8 and 9 to form a double bond, wherein said double bond may optionally be converted to an epoxide.

Particularly preferred are compounds of the formulas

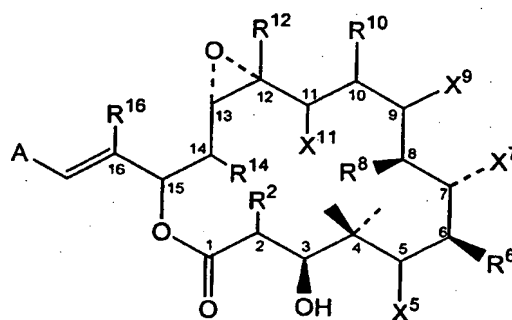


1(a),



1(b)

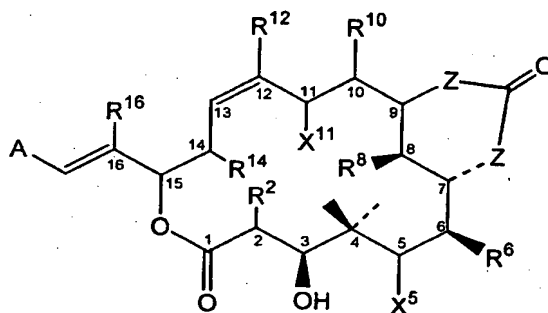
and



1(c)

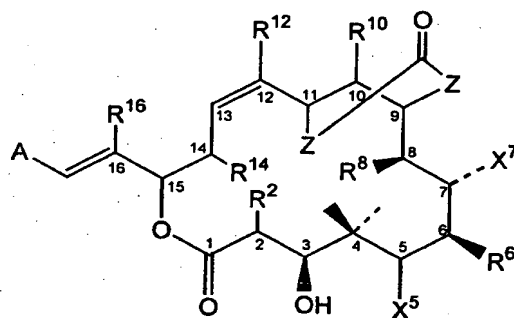
wherein the noted substituents are as defined above.

Especially preferred are compounds of the formulas



1(d)

and



1(e)

wherein both Z are O or one Z is N and the other Z is O, and the remaining substituents are as defined above.

As used herein, a substituent which "comprises an aromatic moiety" contains at least one aromatic ring, such as phenyl, pyridyl, pyrimidyl, thiophenyl, or thiazolyl. The substituent may also include fused aromatic residues such as naphthyl, indolyl, benzothiazolyl, and the like. The aromatic moiety may also be fused to a nonaromatic ring

and/or may be coupled to the remainder of the compound in which it is a substituent through a nonaromatic, for example, alkylene residue. The aromatic moiety may be substituted or unsubstituted as may the remainder of the substituent.

Preferred embodiments of A include the "R" groups shown in Figure 2.

5 As used herein, the term alkyl refers to a C<sub>1</sub>-C<sub>8</sub> saturated, straight or branched chain hydrocarbon radical derived from a hydrocarbon moiety by removal of a single hydrogen atom. Alkenyl and alkynyl refer to the corresponding unsaturated forms. Examples of alkyl include but are not limited to methyl, ethyl, propyl, isopropyl, n-butyl, tert-butyl, neopentyl, i-hexyl, n-heptyl, n-octyl. Lower alkyl (or alkenyl or alkynyl) refers to a 1-4C radical. Methyl is preferred. Acyl refers to alkylCO, alkenylCO or alkynylCO.

10 The terms halo and halogen as used herein refer to an atom selected from fluorine, chlorine, bromine, and iodine. The term haloalkyl as used herein denotes an alkyl group to which one, two, or three halogen atoms are attached to any one carbon and includes without limitation chloromethyl, bromoethyl, trifluoromethyl, and the like.

15 The term heteroaryl as used herein refers to a cyclic aromatic radical having from five to ten ring atoms of which one ring atom is selected from S, O, and N; zero, one, or two ring atoms are additional heteroatoms independently selected from S, O, and N; and the remaining ring atoms are carbon, the radical being joined to the rest of the molecule via any of the ring atoms, such as, for example, pyridyl, pyrazinyl, pyrimidinyl, pyrrolyl, 20 pyrazolyl, imidazolyl, thiazolyl, oxazolyl, isoxazolyl, thiadiazolyl, oxadiazolyl, thiophenyl, furanyl, quinolinyl, isoquinolinyl, and the like.

The term heterocycle includes but is not limited to pyrrolidinyl, pyrazolinyl, pyrazolidinyl, imidazolinyl, imidazolidinyl, piperidinyl, piperazinyl, oxazolidinyl, isoxazolidinyl, morpholinyl, thiazolidinyl, isothiazolidinyl, and tetrahydrofuryl.

25 The term "substituted" as used herein refers to a group substituted by independent replacement of any of the hydrogen atoms thereon with, for example, Cl, Br, F, I, OH, CN, alkyl, alkoxy, alkoxy substituted with aryl, haloalkyl, alkylthio, amino, alkylamino, dialkylamino, mercapto, nitro, carboxaldehyde, carboxy, alkoxycarbonyl, or carboxamide. Any one substituent may be an aryl, heteroaryl, or heterocycloalkyl group.

30 It will apparent that the nature of the substituents at positions 2, 4, 6, 8, 10, 12, 14 and 16 in formula (1) is determined at least initially by the specificity of the AT catalytic domain of modules 9, 8, 7, 6, 5, 4, 3 and 2, respectively. Because AT domains that accept malonyl CoA, methylmalonyl CoA, ethylmalonyl CoA (and in general, lower alkyl

malonyl CoA), as well as hydroxymalonyl CoA, are available, one of the substituents at these positions may be H, and the other may be H, lower alkyl, especially methyl and ethyl, or OH. Further reaction at these positions, e.g., a methyl transferase reaction such as that catalyzed by module 8 of the epothilone PKS, may be used to replace H at these positions as well. Further, an H,OH embodiment may be oxidized to =O or, with the adjacent ring C, be dehydrated to form a  $\pi$ -bond. Both OH and =O are readily derivatized as further described below.

Thus, a wide variety of embodiments of  $R^2$ ,  $R^6$ ,  $R^8$ ,  $R^{10}$ ,  $R^{12}$ ,  $R^{14}$  and  $R^{16}$  is synthetically available. The restrictions set forth with regard to embodiments of these substituents set forth in the definitions with respect to Formula (1) above reflect the information described in the SAR description in Example 8 below.

Similarly,  $\beta$ -carbonyl modifications (or absence of modification) can readily be controlled by modifying the epothilone PKS gene cluster to include the appropriate sequences in the corresponding positions of the epothilone gene cluster which will or will not contain active KR, DH and/or ER domains. Thus, the embodiments of  $X^5$ ,  $X^7$ ,  $X^9$ ,  $X^{11}$  and  $X^{13}$  synthetically available are numerous, including the formation of  $\pi$ -bonds with the adjacent ring positions.

Positions occupied by OH are readily converted to ethers or esters by means well known in the art; protection of OH at positions not to be derivatized may be required. Further, a hydroxyl may be converted to a leaving group, such as a tosylate, and replaced by an amino or halo substituent. A wide variety of "hydroxyl derivatives" such as those discussed above is known in the art.

Similarly, ring positions which contain oxo groups may be converted to "carbonyl derivatives" such as oximes, ketals, and the like. Initial reaction products with the oxo moieties may be further reacted to obtain more complex derivatives. As described in Example 8, such derivatives may ultimately result in a cyclic substituent linking two ring positions.

The enzymes useful in modification of the polyketide initially synthesized, such as transmethylases, dehydratases, oxidases, glycosylation enzymes and the like, can be supplied endogenously by a host cell when the polyketide is synthesized intracellularly, by modifying a host to contain the recombinant materials for the production of these modifying enzymes, or can be supplied in a cell-free system, either in purified forms or as



relatively crude extracts. Thus, for example, the epoxidation of the  $\pi$ -bond at position 12-13 may be effected using the protein product of the *epoK* gene directly *in vitro*.

The nature of A is most conveniently controlled by employing an epothilone PKS which comprises an inactivated module 1 NRPS (using a module 2 substrate) or a KS2 knockout (using a module 3 substrate) as described in Example 6, hereinbelow. Limited variation can be obtained by altering the AT catalytic specificity of the loading module; further variation is accomplished by replacing the NRPS of module 1 with an NRPS of different specificity or with a conventional PKS module. However, at present, variants are more readily prepared by feeding the synthetic module 2 substrate precursors and module 3 substrate precursors to the appropriately altered epothilone PKS as described in Example 6.

#### Pharmaceutical Compositions

The compounds can be readily formulated to provide the pharmaceutical compositions of the invention. The pharmaceutical compositions of the invention can be used in the form of a pharmaceutical preparation, for example, in solid, semisolid, or liquid form. This preparation will contain one or more of the compounds of the invention as an active ingredient in admixture with an organic or inorganic carrier or excipient suitable for external, enteral, or parenteral application. The active ingredient may be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers for tablets, pellets, capsules, suppositories, pessaries, solutions, emulsions, suspensions, and any other form suitable for use.

The carriers which can be used include water, glucose, lactose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea, and other carriers suitable for use in manufacturing preparations, in solid, semi-solid, or liquified form. In addition, auxiliary stabilizing, thickening, and coloring agents and perfumes may be used. For example, the compounds of the invention may be utilized with hydroxypropyl methylcellulose essentially as described in U.S. Patent No. 4,916,138, incorporated herein by reference, or with a surfactant essentially as described in EPO patent publication No. 428,169, incorporated herein by reference.

Oral dosage forms may be prepared essentially as described by Hondo *et al.*, 1987, Transplantation Proceedings XIX, Supp. 6: 17-22, incorporated herein by reference. Dosage forms for external application may be prepared essentially as described in EPO

patent publication No. 423,714, incorporated herein by reference. The active compound is included in the pharmaceutical composition in an amount sufficient to produce the desired effect upon the disease process or condition.

5 For the treatment of conditions and diseases caused by infection, immune system disorder (or to suppress immune function), or cancer, a compound of the invention may be administered orally, topically, parenterally, by inhalation spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvant, and vehicles. The term parenteral, as used herein, includes subcutaneous injections, and intravenous, intrathecal, intramuscular, and intrasternal injection or  
10 infusion techniques.

Dosage levels of the compounds of the present invention are of the order from about 0.01 mg to about 100 mg per kilogram of body weight per day, preferably from about 0.1 mg to about 50 mg per kilogram of body weight per day. The dosage levels are useful in the treatment of the above-indicated conditions (from about 0.7 mg to about 3.5  
15 mg per patient per day, assuming a 70 kg patient). In addition, the compounds of the present invention may be administered on an intermittent basis, i.e., at semi-weekly, weekly, semi-monthly, or monthly intervals.

The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular  
20 mode of administration. For example, a formulation intended for oral administration to humans may contain from 0.5 mg to 5 gm of active agent compounded with an appropriate and convenient amount of carrier material, which may vary from about 5 percent to about 95 percent of the total composition. Dosage unit forms will generally contain from about 0.5 mg to about 500 mg of active ingredient. For external administration, the compounds  
25 of the invention may be formulated within the range of, for example, 0.00001% to 60% by weight, preferably from 0.001% to 10% by weight, and most preferably from about 0.005% to 0.8% by weight.

It will be understood, however, that the specific dose level for any particular patient will depend on a variety of factors. These factors include the activity of the specific  
30 compound employed; the age, body weight, general health, sex, and diet of the subject; the time and route of administration and the rate of excretion of the drug; whether a drug combination is employed in the treatment; and the severity of the particular disease or condition for which therapy is sought.

A detailed description of the invention having been provided above, the following examples are given for the purpose of illustrating the present invention and shall not be construed as being a limitation on the scope of the invention or claims.

5

### Example 1

#### DNA Sequencing of Cosmid Clones and Subclones Thereof

The epothilone producing strain, *Sorangium cellulosum* SMP44, was grown on a cellulose-containing medium, see Bollag *et al.*, 1995, Cancer Research 55: 2325-2333, incorporated herein by reference, and epothilone production was confirmed by LC/MS analysis of the culture supernatant. Total DNA was prepared from this strain using the procedure described by Jaoua *et al.*, 1992, Plasmid 28: 157-165, incorporated herein by reference. To prepare a cosmid library, *S. cellulosum* genomic DNA was partially digested with Sau3AI and ligated with BamHI-digested pSupercos (Stratagene). The DNA was packaged in lambda phage as recommended by the manufacturer and the mixture then used to infect *E. coli* XL1-Blue MR cells. This procedure yielded approximately 3,000 isolated colonies on LB-ampicillin plates. Because the size of the *S. cellulosum* genome is estimated to be circa  $10^7$  nucleotides, the DNA inserts present among 3000 colonies would correspond to circa 10 *S. cellulosum* genomes.

To screen the library, two segments of KS domains were used to design oligonucleotide primers for a PCR with *Sorangium cellulosum* genomic DNA as template. The fragment generated was then used as a probe to screen the library. This approach was chosen, because it was found, from the examination of over a dozen PKS genes, that KS domains are the most highly conserved (at the amino acid level) of all the PKS domains examined. Therefore, it was expected that the probes produced would detect not only the epothilone PKS genes but also other PKS gene clusters represented in the library. The two degenerate oligonucleotides synthesized using conserved regions within the ketosynthase (KS) domains compiled from the DEBS and soraphen PKS gene sequences were (standard nomenclature for degenerate positions is used): CTSGTSKCSSTBCACCTSGCSTGC and TGAYRTGSGCGTTSGTSCCGSWGA. A single band of ~750 bp, corresponding to the predicted size, was seen in an agarose gel after PCR employing the oligos as primers and *S. cellulosum* SMP44 genomic DNA as template. The fragment was removed from the gel and cloned in the HincII site of pUC118 (which is a derivative of pUC18 with an insert sequence for making single stranded DNA). After transformation of *E. coli*, plasmid DNA

from ten independent clones was isolated and sequenced. The analysis revealed nine unique sequences that each corresponded to a common segment of KS domains in PKS genes. Of the nine, three were identical to a polyketide synthase gene cluster previously isolated from this organism and determined not to belong to the epothilone gene cluster  
5 from the analysis of the modules. The remaining six KS fragments were excised from the vector, pooled, end-labeled with  $^{32}\text{P}$  and used as probe in hybridizations with the colonies containing the cosmid library under high stringency conditions.

The screen identified 15 cosmids that hybridized to the pooled KS probes. DNA was prepared from each cosmid, digested with NotI, separated on an agarose gel, and  
10 transferred to a nitrocellulose membrane for Southern hybridization using the pooled KS fragments as probe. The results revealed that two of the cosmids did not contain KS-hybridizing inserts, leaving 13 cosmids to analyze further. The blot was stripped of the label and re-probed, under less stringent conditions, with labeled DNA containing the sequence corresponding to the enoylreductase domain from module four of the DEBS  
15 gene cluster. Because it was anticipated that the epothilone PKS gene cluster would encode two consecutive modules that contain an ER domain, and because not all PKS gene clusters have ER domain-containing modules, hybridization with the ER probe was predicted to identify cosmids containing insert DNA from the epothilone PKS gene cluster. Two cosmids were found to hybridize strongly to the ER probe, one hybridized  
20 moderately, and a final cosmid hybridized weakly. Analysis of the restriction pattern of the NotI fragments indicated that the two cosmids that hybridized strongly with the ER probe overlapped one another. The nucleotide sequence was also obtained from the ends of each of the 13 cosmids using the T7 and T3 primer binding sites. All contained sequences that showed homology to PKS genes. Sequence from one of the cosmids that  
25 hybridized strongly to the ER probe showed homology to NRPSs and, in particular, to the adenylation domain of an NRPS. Because it was anticipated that the thiazole moiety of epothilone might be derived from the formation of an amide bond between an acetate and cysteine molecule (with a subsequent cyclization step), the presence of an NRPS domain in a cosmid that also contained ER domain(s) supported the prediction that this cosmid  
30 might contain all or part of the epothilone PKS gene cluster.

Preliminary restriction analysis of the 12 remaining cosmids suggested that three might overlap with the cosmid of interest. To verify this, oligonucleotides were synthesized for each end of the four cosmids (determined from the end sequencing

described above) and used as primer sets in PCRs with each of the four cosmid DNAs. Overlap would be indicated by the appearance of a band from a non-cognate primer-template reaction. The results of this experiment verified that two of the cosmids overlapped with the cosmid containing the NRPS. Restriction mapping of the three  
5 cosmids revealed that the cosmids did, in fact, overlap. Furthermore, because PKS sequences extended to the end of the insert in the last overlapping fragment, based on the assumption that the NRPS would map to the 5'-end of the cluster, the results also indicated that the 3' end of the gene cluster had not been isolated among the clones identified.

To isolate the remaining segment of the epothilone biosynthesis genes, a PCR  
10 fragment was generated from the cosmid containing the most 3'-terminal region of the putative gene cluster. This fragment was used as a probe to screen a newly prepared cosmid library of *Sorangium cellulosum* genomic DNA of again approximately 3000 colonies. Several hybridizing clones were identified; DNA was made from six of them. Analysis of NotI-digested fragments indicated that all contained overlapping regions. The  
15 cosmid containing the largest insert DNA that also had the shortest overlap with the cosmid used to make the probe was selected for further analysis.

Restriction maps were created for the four cosmids, as shown in Figure 1. Sequence obtained from one of the ends of cosmid pKOS35-70.8A3 showed no homology to PKS sequences or any associated modifying enzymes. Similarly, sequence from one  
20 end of cosmid pKOS35-79.85 also did not contain sequences corresponding to a PKS region. These findings supported the observation that the epothilone cluster was contained within the ~70 kb region encompassed by the four cosmid inserts.

To sequence the inserts in the cosmids, each of the NotI restriction fragments from the four cosmids was cloned into the NotI site of the commercially available pBluescript  
25 plasmid. Initial sequencing was performed on the ends of each of the clones. Analysis of the sequences allowed the prediction, before having the complete sequence, that there would be 10 modules in this PKS gene cluster, a loading domain plus 9 modules.

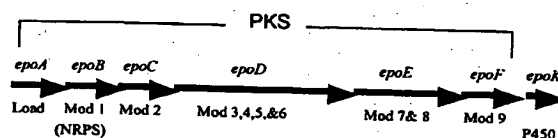
Sequence was obtained for the complete PKS as follows. Each of the 13 non-overlapping NotI fragments was isolated and subjected to partial HinPI digestion.  
30 Fragments of ~2 to 4 kb in length were removed from an agarose gel and cloned in the AccI site of pUC118. Sufficient clones from each library of the NotI fragments were sequenced to provide at least 4 -fold coverage of each. To sequence across each of the NotI sites, a set of oligos, one 5' and the other 3' to each NotI site, was made and used as

primers in PCR amplification of a fragment that contained each NotI site. Each fragment produced in this manner was cloned and sequenced.

The nucleotide sequence was determined for a linear segment corresponding to ~72 kb. Analysis revealed a PKS gene cluster with a loading domain and nine modules.

5 Downstream of the PKS sequence is an ORF, designated *epoK*, that shows strong homology to cytochrome P450 oxidase genes and encodes the epothilone epoxidase. The nucleotide sequence of 15 kb downstream of *epoK* has also been determined: a number of additional ORFs have been identified but an ORF that shows homology to any known dehydratase has not been identified. The *epoL* gene may encode a dehydratase activity, but  
 10 this activity may instead be resident within the epothilone PKS or encoded by another gene.

The PKS genes are organized in 6 open reading frames. At the polypeptide level, the loading domain and modules 1, 2, and 9 appear on individual polypeptides; their corresponding genes are designated *epoA*, *epoB*, *epoC* and *epoF* respectively. Modules 3,  
 15 4, 5, and 6 are contained on a single polypeptide whose gene is designated *epoD*, and modules 7 and 8 are on another polypeptide whose gene is designated *epoE*. It is clear from the spacing between ORFs that *epoC*, *epoD*, *epoE* and *epoF* constitute an operon. The *epoA*, *epoB*, and *epoK* gene may be also part of the large operon, but there are spaces of approximately 100 bp between *epoB* and *epoC* and 115 bp between *epoF* and *epoK*  
 20 which could contain a promoter. The present invention provides the intergenic sequences in recombinant form. At least one, but potentially more than one, promoter is used to express all of the epothilone genes. The epothilone PKS gene cluster is shown schematically below.



25 A detailed examination of the modules shows an organization and composition that is consistent with one able to be used for the biosynthesis of epothilone. The description that follows is at the polypeptide level. The sequence of the AT domain in the loading module and in modules 3, 4, 5, and 9 shows similarity to the consensus sequence for malonyl loading domains, consistent with the presence of an H side chain at C-14, C-12

(epothilones A and C), C-10, and C-2, respectively, as well as the loading region. The AT domains in modules 2, 6, 7, and 8 resemble the consensus sequence for methylmalonyl specifying AT domains, again consistent with the presence of methyl side chains at C-16, C-8, C-6, and C-4 respectively.

5       The loading module contains a KS domain in which the cysteine residue usually present at the active site is instead a tyrosine. This domain is designated as KS<sup>y</sup> and serves as a decarboxylase, which is part of its normal function, but cannot function as a condensing enzyme. Thus, the loading domain is expected to load malonyl CoA, move it to the ACP, and decarboxylate it to yield the acetyl residue required for condensation with  
10       cysteine.

      Module 1 is the non-ribosomal peptide synthetase that activates cysteine and catalyzes the condensation with acetate on the loading module. The sequence contains segments highly similar to ATP-binding and ATPase domains, required for activation of amino acids, a phosphopantotheinylation site, and an elongation domain. In database  
15       searches, module 1 shows very high similarity to a number of previously identified peptide synthetases.

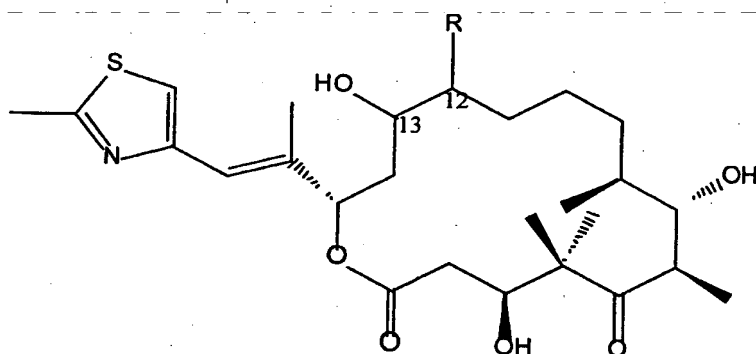
      Module 2 determines the structure of epothilone at C-15 – C-17. The presence of the DH domain in module 2 yields the C-16-17 dehydro moiety in the molecule. The domains in module 3 are consistent with the structure of epothilone at C-14 and C-15; the  
20       OH that comes from the action of the KR is employed in the lactonization of the molecule.

      Module 4 controls the structure at C-12 and C-13 where a double bond is found in epothilones C and D, consistent with the presence of a DH domain. Although the sequence of the AT domain appears to resemble those that specify malonate loading, it can also load methylmalonate, thereby accounting in part for the mixture of epothilones found in the  
25       fermentation broths of the naturally producing organisms.

      A significant departure from the expected array of functions was found in module 4. This module was expected to contain a DH domain, thereby directing the synthesis of epothilones C and D as the products of the PKS. Rigorous analysis revealed that the space between the AT and KR domains of module 4 was not large enough to accommodate a  
30       functional DH domain. Thus, the extent of reduction at module 4 does not proceed beyond the ketoreduction of the beta-keto formed after the condensation directed by module 4. Because the C-12,13 unsaturation has been demonstrated (epothilones C and D), there must be an additional dehydratase function that introduces the double bond, and this

function is believed to be in the PKS itself or resident in an ORF in the epothilone biosynthetic gene cluster.

Thus, the action of the dehydratase could occur either during the synthesis of the polyketide or after cyclization has taken place. In the former case, the compounds  
5 produced at the end of acyl chain growth would be epothilones C and D. If the C-12,13 dehydration were a post-polyketide event, the completed acyl chain would have a hydroxyl group at C-13, as shown below. The names epothilones G and H have been assigned to the 13-hydroxy compounds produced in the absence of or prior to the action of the dehydratase.



10  
Epothilones G (R=H) and H (R=CH<sub>3</sub>).

Modules 5 and 6 each have the full set of reduction domains (KR, DH and ER) to yield the methylene functions at C-11 and C-9. Modules 7 and 9 have KR domains to yield the hydroxyls at C-7 and C-3, and module 8 does not have a functional KR domain,  
15 consistent with the presence of the keto group at C-5. Module 8 also contains a methyltransferase (MT) domain that results in the presence of the geminal dimethyl function at C-4. Module 9 has a thioesterase domain that terminates polyketide synthesis and catalyzes ring closure. The genes, proteins, modules, and domains of the epothilone PKS are summarized in the Table hereinabove.

20 Inspection of the sequence has revealed translational coupling between *epoA* and *epoB* (loading domain and module 1) and between *epoC* and *epoD*. Very small gaps are seen between *epoD* and *epoE* and *epoE* and *epoF* but gaps exceeding 100 bp are found between *epoB* and *epoC* and *epoF* and *epoK*. These intergenic regions may contain promoters. Sequencing efforts have not revealed the presence of regulatory genes, and it is  
25 possible that epothilone synthesis is not regulated by operon specific regulation in *Sorangium cellulosum*.



The sequence of the epothilone PKS and flanking regions has been compiled into a single contig, as shown below.

```

1  TCGTGCGCGG GCACGTCGAG GCGTTTGCCG ACTTCGGCGG CGTCCCGCGC GTGCTGCTCT
5  61  ACGACAACCT CAAGAACGCC GTCGTCGAGC GCCACGGCGA CGCGATCCGG TTCCACCCCA
121 CGCTGCTGGC TCTGTCGGCG GATTACCGCT TCGAGCCGCG CCCCCTCGCC GTGCCCCGCG
181 GCAACGAGAA GGGCCGCGTC GAGCGCGCCA TCCGCTACGT CCGCGAGGGC TTCTTCGAGG
241 CCCGGGCCTA CGCCGACCTC GGAGACCTCA ACCGCCAAGC GACCGAGTGG ACCAGCTCCG
301 CGGCGCTCGA TCGCTCCTGG GTCGAGGACC GCGCCCGCAC CGTGCGTCAG GCCTTCGACG
10 361 ACGAGCGCAG CGTGCTGCTG CGATCCGCTG ACACACCGTT TCCGGACCAC GAGCGCGTCC
421 AGGTCGAGGT CGGAAAGACC CCCTACGCGC GCTTCGATCT CAACGACTAC TCGGTCCCCC
481 ACGACCGGAC GCGCCGCACG CTGGTCGTCC TCGCCGACCT CAGTCAGGTA CGCATCGCCG
541 ACGGCAACCA GATCGTCGCG ACCACGTCC GTTCGTGGGA CCGCGGCCAG CAGATCGAGC
601 AGCCCGAGCA CCTCCAGCGC CTGGTCGACG AGAAGCGCCG CGCCCGCGAG CACCGCGGCC
661 TTGATCGCCT CGCGCGCGCC GCCCGCAGCA GCCAGGCATT CCTGCGCATC GTCGCCGAGC
15 721 GCGGCGATAA CGTCGGCAGC GCGATCGCCC GGCTTCTGCA ACTGCTCGAC GCCGTGGGCG
781 CCGCCGAGCT CGAAGAGGCC CTGGTCGAGG TGCTTGAGCG CGACACCATC CACATCGGTG
841 CCGTCCGCCA GGTGATCGAC CGCCGCGCGT CCGAGCGCCA CCTGCCGCTC CCAGTCTCAA
901 TCCCCGTCAC CCGCGGCGAG CACGCCGCC TCGTCGTAC GCCGCATTCC CTCACCACCT
961 ACGACGCCCT GAAGAAGGAC CCGACGCCAT GACCGACCTG ACGCCACCG AGACCAAAGA
20 1021 CCGGCTCAAG AGCCTCGGCC TCTTCGGCCT GCTCGCCTGC TGGGAGCAGC TCGCCGACAA
1081 GCCCTGGCTT CGCGAGGTGC TCGCCATCGA GGAGCGCGAG CGCCACAAGC GCAGCCTCGA
1141 ACGCCGCTG AAGAACTCCC GCGTCGCCGC CTTCAAGCCC ATGACCGACT TCGACTCGTC
1201 CTGCCCCAAG AAGATCGACC GCGAGGCCGT CGACGACCTC TACGATAGCC GCTACGCGGA
1261 CCTGCTCTTC GAGGTGCTCA CCCGTCGCTA CGACGCGCAG AAGCCGCTCT TGCTCAGCAC
25 1321 GAACAAGGCA TTCGCCGACT GGGGCCAGGT CTTCCCGCAC GCCCGGTGCG TCGTCACGCT
1381 CGTCGACCGG CTCGTGCACC GCGCCGAGGT GATCGAGATC GAGGCCGAGA GCTACCGGCT
1441 GAAGGAAGCC AAGGAGCTCA ACGCCACCCG CACCAAGCAG CGCCGACCA AGAAGCACTG
1501 AGCGGCATTT TCACCGGTGA ACTTCACCGA AATCCCGCGT GTTGCCGAGA TCATCTACAG
1561 GCGGATCGAG ACCGTGCTCA CGGCGTGGAC GACATGGCGC GGAAACGTCG TCGTAACTGC
30 1621 CCAGCAATGT CATGGGAATG GCCCCTTGAG GGGCTGGCCG GGGTCGACGA TATCGCGCGA
1681 TCTCCCCGTC AATTCCCGAG CGTAAAAGAA AAATTTGTCA TAGATCGTAA GCTGTGCTAG
1741 TGATCTGCCT TACGTTACGT CTTCCGCACC TCGAGCGAAT TCTCTCGGAT AACTTTCAAG
1801 TTTTCTGAGG GGGCTTGGTC TCTGGTTCTT CAGGAAGCCT GATCGGGACG AGCTAATTCC
1861 CATCCATTTT TTTGAGACTC TGCTCAAAGG GATTAGACCG AGTGAGACAG TTCTTTTGCA
35 1921 GTGAGCGAAG AACCTGGGGC TCGACCGGAG GACGATCGAC GTCCGCGAGC GGGTCAGCCG
1981 CTGAGGATGT GCCCGTCGTG GCGGATCGTC CCATCGAGCG CGCAGCCGAA GATCCGATTG
2041 CGATCGTCGG AGCGGGCTGC CGTCTGCCCC GTGGCGTGAT CGATCTGAGC GGGTTCTGGA
2101 CGCTCCTCGA GGGCTCGCGC GACACCGTCG GGCAAGTCCC CGCCGAACGC TGGGATGCAG
40 2161 CAGCGTGGTT TGATCCCGAC CTCGATGCCC CGGGGAAGAC GCCCGTTACG CGCGCATCTT
2221 TCCTGAGCGA CGTAGCCTGC TTCGACGCCT CTTCTTTCGG CATCTCGCCT CGCGAAGCGC
2281 TGCGGATGGA CCCTGCACAT CGACTCTTGC TGGAGGTGTG CTGGGAGGCG CTGGAGAACG
2341 CCGCGATCGC TCCATCGGCG CTCGTGCGTA CGGAAACGGG AGTGTTTCATC GGGATCGGCC
2401 CGTCCGAATA TGAGGCCGCG CTGCCGCGAG CGACGGCGTC CGCAGAGATC GACGCTCATG
45 2461 GCGGGCTGGG GACGATGCCC AGCGTCGGAG CGGGCCGAAT CTCGTATGTC CTCGGGCTGC
2521 GAGGGCCGTG TGTGCGGGTG GATACGGCCT ATTCGTCCTC GCTCGTGGCC GTTCATCTGG
2581 CCTGTGAGAG CTTGCGCTCC GGGGAATGCT CCACGGCCCT GGCTGGTGGG GTATCGCTGA
2641 TGTTGTGCGC GAGCACCTC GTGTGGCTCT CGAAGACCCG CGCGCTGGCC ACGGACGGTC
2701 GCTGCAAGGC GTTTTCGGCG GAGGCCGATG GGTTCGGACG AGGCGAAGGG TGCGCCGTGC
50 2761 TGGTCTCTCA GCGGCTCAGT GGAGCCCGCG CGGACGGCGA CCGGATATTG GCGGTGATTC
2821 GAGGATCCGC GATCAATCAC GACGGAGCGA GCAGCGGTCT GACCGTGCCG AACGGGAGCT
2881 CCCAAGAAAT CGTGCTGAAA CGGGCCCTGG CGGACGCAGG CTGCGCCGCG TCTTCGGTGG
2941 GTTATGTGCG GGCACACGGC ACGGGCACGA CGCTTGGTGA CCCCATCGAA ATCCAAGCTC
3001 TGAATGCGGT ATACGGCCTC GGGCGAGACG TCGCCACGCC GCTGCTGATC GGGTCGGTGA
3061 AGACCAACCT TGGCCATCCT GAGTATGCGT CGGGGATCAC TGGGCTGCTG AAGGTGCTCT
55 3121 TGTCCCTTCA GCACGGCGAG ATTCTGCGC ACCTCCACGC GCAGGCGCTG AACCCCGGGA
3181 TCTCATGGGG TGATCTTCGG CTGACCTGCA CGCGCGCCCG GACACCGTGG CCGGACTGGA
3241 ATACGCCGCG ACGGGCGGGG GTGAGCTCGT TCGGCATGAG CCGGACCAAC CCGCACTGG
3301 TGCTGGAAGA GGCGCCGGCG GCGACGTGCA CACCGCCGGC GCCGGAGCGG CCGGCAGAGC

```

3361 TGCTGGTGCT GTCGGCAAGG ACCGCGGCAG CTTTGGATGC ACACGCGGCG CGGCTGCGCG  
3421 ACCATCTGGA GACCTACCCT TCGCAGTGTC TGGGCGATGT GGCCTTCAGT CTGGCGACGA  
3481 CGCGCAGCGC GATGGAGCAC CGGCTCGCGG TGGCGGCGAC GTCGAGCGAG GGGCTGCGGG  
5 3541 CAGCCCTGGA CGCTGCGGCG CAGGGACAGA CGCCGCCCGG TGTGGTGCGC GGTATCGCCG  
3601 ATTCTCTACG CGGCAAGCTC GCCTTTCTCT TCACCGGACA GGGGGCGCAG ACGCTGGGCA  
3661 TGGGCCGTGG GCTGTATGAT GTATGGCCCG CGTTCCGCGA GCGGTTTCAG CTGTGCGTGA  
3721 GGCTGTTCAA CCAGGAGCTC GACCGGCCGC TCCGCGAGGT GATGTGGGCC GAACCGGCCA  
3781 GCGTCGACGC CGCGCTGCTC GACCAGACAG CCTTTACCCA GCCGCGCTG TTCACCTTCG  
10 3841 AGTATGCGCT CGCCGCGCTG TGGCGGTCTG GGGGCGTAGA GCCGGAGTTG GTCGCTGGCC  
3901 ATAGCATCGG TGAGCTGGTG GCTGCCTGCG TGGCGGGCGT GTTCTCGCTT GAGGACGCGG  
3961 TGTTCCTGGT GGCTGCGCGC GGGCGCCTGA TGCAGGCGCT GCCGCGCGGC GGGGCGATGG  
4021 TGTCGATCGC GCGCGTCAAC GGTCCGACCG AGGTGGTCAAT CGCGGCGGCC GGGCAACCCG  
4081 TGTCGATCGC CGCGGTCAAC GGTCCGACCG AGGTGGTCAAT CGCGGCGGCC GGGCAACCCG  
15 4141 TGCATGCGAT CGCGCGCGCG ATGGCCGCGC GCGGGGCGCG AACCAAGGCG CTCACGTCT  
4201 CGCATGCGTT CCACTACCCG CTCATGGCCC CGATGCTGGA GCGGTTCCGG CGTGTGGCCG  
4261 AGTCGGTGAG CTACCGGCGG CCGTCGATCG TCCTGGTCAG CAATCTGAGC GGGGAAGGCTG  
4321 GCACAGACGA GGTGAGCTCG CCGGGCTATT GGGTGCGCCA CGCGCGAGAG GTGGTGCGCT  
4381 TCGCGGATGG AGTGAAGGCG CTGCACGCGG CCGGTGCGGG CACCTTCGTC GAGGTCGGTC  
20 4441 CGAAATCGAC GCTGCTCGGC CTGGTGCCTG CCGGCGCTGC CCGGCGCTGC  
4501 TCGCATCGTC GCGCGCTGGG CGTGACGAGC CAGCGACCGT GCTCGAGGCG CTCGCGCGGC  
4561 TCTGGGCCGT CCGTGCCCTG GTCTCCTGGG CCGGCCTCTT CCCCTCAGGG GGGCGCGGGG  
4621 TGCCGCTGCC CACGTACCCT TGGCAGCGCG AGCGCTACTG GATCGACACG AAAGCCGACG  
4681 ACGCGGCGCG TGGCGACCGC CGTGCTCCGG GAGCGGGTCA CGACGAGGTC GAGAAGGGGG  
25 4741 GCGCGGTGCG CGGCGGCGAC CCGCGCAGCG CTCGGCTCGA CCATCCGCGG CCCGAGAGCG  
4801 GACGCCGGA GAAGGTCGAG GCGCGCGCG ACCGTCCGTT CCGGCTCGAG ATCGATGAGC  
4861 CAGGCGTGCT CGATCGCCTG GTGCTTCGGG TCACGAGCG GCGCGCCCCT GTCTTGGCG  
4921 AGGTGAGAT CGCCGTCGAC GCGGCGGGGC TCAGCTTCAA TGATGTCCAG CTCGCGCTGG  
4981 GCATGGTGCC CGACGACCTG CCGGGAAGC CCAACCCTCC GCTGCTGCTC GGAGGCGAGT  
30 5041 GCGCCGGGCG CATCGTCGCC GTGGGCGAGG GCGTGAACGG CCTTGTGGTG GGCCAACCGG  
5101 TCATCGCCCT TTCGGCGGGA GCGTTTGCTA CCCACGTAC CAGCTCGGCT GCGCTGGTGC  
5161 TGCTCGGCC TCAGGCGCTC TCGGCGACCG AGGCGGCCG CATGCCCGTC GCGTGGTGA  
5221 CGGCATGGTA CGCGCTCGAC GGAATAGCCC GCCTTCAGCC GGGGGAGCGG GTGCTGATCC  
5281 ACGCGGCGAC CGGCGGGGTC GGTCTCGCCG CCGTGCACTG GCGCGAGCAC GTGGGAGCCG  
35 5341 AGGTCCATGC GACGGCCGCG ACGCCGAGA AGCGCGCCTA CCTGGAGTCG CTGGGCGTGC  
5401 GGTATGTGAG CGATTCCCGC TCGGACCGGT TCGTCCCGA CGTGCGCGCG TGGACGGGCG  
5461 GCGAGGGAGT AGACGTCGTG CTCAACTCGC TTTCCGGGCGA GCTGATCGAC AAGAGTTTCA  
5521 ATCTCCTGCG ATCGCACGGC CGGTTTGTGG AGCTCGGCAA GCGCGACTGT TACGCGGATA  
5581 ACCAGCTCGG GCTGCGGCCG TTCCTGCGCA ATCTCTCCTT CTCGCTGGTG GATCTCCGGG  
40 5641 GGATGATGCT CGAGCGGCCG GCGCGGGTCC GTGCGCTCTT CGAGGAGCTC CTCGGCCTGA  
5701 TCGCGGCGAG CGTGTTCACC CCTCCCCCA TCGCGACGCT CCCGATCGCT CGTGTGCGCG  
5761 ATGCGTTCCG GAGCATGGCG GAGCGCGACG ATCTTGGGAA GCTCGTACTC ACGCTGGGTG  
5821 ACCCGGAGGT CCAGATCCGT ATTCCGACCC ACGCAGGCGC CGGCCCCGTC ACCGGGGATC  
5881 GGGATCTGCT CGACAGGCTC GCGTCAGCTG CGCCGGCCGC GCGCGCGGCG GCGCTGGAGG  
45 5941 CGTTCCCTCCG TACGCAGGTC TCGCAGGTGC TCGCACGCC CGAAATCAAG GTCGGCGCGG  
6001 AGGCGCTGTT CACCCGCTC GGCATGGACT CGCTCATGGC CGTGGAGCTG CGCAATCGTA  
6061 TCGAGGCGAG CCTCAAGCTG AAGCTGTGCA CGACGTTCTT GTCCACGTCC CCAATATCG  
6121 CTTGTTGAC CCAAAACCTG TTGGATGCTC TCGCCACAGC TCTCTCCTTG GAGCGGGTGG  
6181 CGGCGGAGAA CCTACGGGCA GCGGTGCAA GCGACTTCGT CTCATCGGGC GCAGATCAAG  
50 6241 ACTGGGAAAT CATTGCCCTA TGACGATCAA TCAGCTTCTG AACGAGCTCG AGCACCAGGG  
6301 GTCAAGCTG GCGGCCGATG GGGAGCGCCT CCAGATACAG GCGGGGAGG ACCGCCGTGA  
6361 TCGCAACCTG CTCGCTCGAA TCTCCGAGCA CAAAAGCACG ATCCTGACGA TGCTCCGTCA  
6421 GAGACTCCCC GCAGAGTCCA TCGTGCCCG CCCAGCCGAG CCGCACGTTT CCGTTCTCT  
6481 CACAGACATC CAAGGATCCT ACTGGCTGGG TCGGACAGGA GCGTTTACGG TCCCCAGCGG  
55 6541 GATCCACGCC TATCGCGAAT ACGACTGTAC GGATCTCGAC GTGGCGAGGC TGAGCCGCGC  
6601 CTTTCGGAAA GTCGTCGCGC GGCACGACAT GCTTCGGGCC CACACGCTGC CCGACATGAT  
6661 GCAGGTGATC GAGCCTAAAG TCGACGCCGA CATCGAGATC ATCGATCTGC GCGGGCTCGA  
6721 CCGGAGCACA CGGGAAGCGA GGCTCGTATC GTTGCAGAT GCGATGTGCG ACCGCATCTA  
6781 TGACACCGAG CGCCCTCCGC TCTATCACGT CGTCCGCGTT CCGCTGGAGC AGCAGCAAAC  
6841 CCGTCTCGTG CTCAGTATCG ATCTCATTA CTTGACCTA GGCAGCCTGT CCATCATCTT

	6901	CAAGGATTGG	CTCAGCTTCT	ACGAAGATCC	CGAGACCTCT	CTCCCTGTCC	TGGAGCTCTC
	6961	GTACCGCGAC	TATGTGCTCG	CGCTGGAGTC	TCGCAAGAAG	TCTGAGGCGC	ATCAACGATC
	7021	GATGGATTAC	TGGAAGCGGC	GCGTCGCCGA	GCTCCCACCT	CCGCCGATGC	TTCCGATGAA
5	7081	GGCCGATCCA	TCTACCCCTGA	GGGAGATCCG	CTTCCGGCAC	ACGGAGCAAT	GGCTGCCGTC
	7141	GGACTCCTGG	AGTCGATTGA	AGCAGCGTGT	CGGGGAGCGC	GGGCTGACCC	CGACGGGCGT
	7201	CATTCTGGCT	GCATTTTCCG	AGGTGATCGG	GCGCTGGAGC	GCGAGCCCCC	GGTTTACGCT
	7261	CAACATAACG	CTCTTCAACC	GGCTCCCCGT	CCATCCGCGC	GTGAACGATA	TCACCGGGGA
	7321	CTTCACGTCG	ATGGTCCTCC	TGGACATCGA	CACCACTCGC	GACAAGAGCT	TCGAACAGCG
10	7381	CGCTAAGCGT	ATTCAAGAGC	AGCTGTGGGA	AGCGATGGAT	CACTGCGACG	TAAGCGGTAT
	7441	CGAGGTCCAG	CGAGAGGCCG	CCCCGGTCTT	GGGGATCCAA	CGAGGCGCAT	TGTTCCCCGT
	7501	GGTGCTCACG	AGCGCGCTCA	ACCAGCAAGT	CGTTGGTGTC	ACCTCGCTGC	AGAGGCTCGG
	7561	CACTCCGGTG	TACACCAGCA	CGCAGACTCC	TCAGCTGCTG	CTGGATCATC	AGATCTACGA
	7621	GCACGATGGG	GACCTCGTCC	TCGCGTGGGA	CATCGTCGAC	GGAGTGTTCC	CGCCCGACCT
15	7681	TCTGGACGAC	ATGCTCGAAG	CGTACGTCGC	TTTTCTCCGG	CGGCTCACTG	AGGAACCATG
	7741	GAGTGAACAG	ATGCGCTGTT	CGCTTCCGCC	TGCCAGCTA	GAAAGCGGGG	CGAGCGCAAA
	7801	CGAGACCAAC	TCGCTGCTGA	GCGAGCATAC	GCTGCACGGC	CTGTTGCGCG	CGCGGGTCGA
	7861	GCAGCTGCCCT	ATGCAGCTCG	CCGTGGTGTC	GGCGCGCAAG	ACGCTCACGT	ACGAAGAGCT
	7921	TTTCGCGCCGT	TCGCGGCGAC	TTGGCGCGCG	GCTGCGCGAG	CAGGGGGCAC	GCCCCAACAC
	7981	ATTGGTCGCG	GTGGTGATGG	AGAAAGGCTG	GGAGCAGGTT	GTCGCGGTTT	TCGCGGTGCT
20	8041	CGAGTCAGGC	GCGGCCTACG	TGCCGATCGA	TGCCGACCTA	CCGCGGGAGC	GTATCCACTA
	8101	CCTCCTCGAT	CATGGTGAGG	TAAAGCTCGT	GCTGACGCAG	CCATGGCTGG	ATGGCAAACCT
	8161	GTCATGGCCG	CCGGGGATCC	AGCGGCTGCT	CGTGAGCGAT	GCCGGCGTGC	AAGGCGACGG
	8221	CGACCAGCTT	CCGATGATGC	CCATTACAGC	ACCTTCGGAT	CTCGCGTATG	TCATCTACAC
25	8281	CTCGGGATCC	ACAGGGTTGC	CCAAGGGGGT	GATGATCGAT	CATCGGGGTG	CCGTCAACAC
	8341	CATCCTGGAC	ATCAACGAGC	GCTTCGAAAT	AGGGCCCCGA	GACAGAGTGC	TGGCGCTCTC
	8401	CTCGCTGAGC	TTGATCTCT	CGGTCTACGA	TGTGTTCCGG	ATCCTGGCGG	CGGGCGGTAC
	8461	GATCGTGGTG	CCGGACGCGT	CCAAGCTGCG	CGATCCGGCG	CATTGGGCAG	CGTTGATCGA
	8521	ACGAGAGAAG	GTGACGGTGT	GGAACTCGGT	GCCGGCGCTG	ATGCGGATGC	TCGTGAGCA
30	8581	TTCCGAGGGT	CGCCCCGATT	CGCTCGCTAG	GTCTCTGCGG	CTTTCGCTGC	TGAAGCGCGA
	8641	CTGGATCCCG	GTGGGCCTGC	CTGGCGAGCT	CCAGGCCATC	AGGCCCGGCG	TGTCGGTGAT
	8701	CAGCCTGGGC	GGGGCCACCG	AAGCGTCGAT	CTGGTCCATC	GGGTACCCCG	TGAGGAACGT
	8761	CGATCCATCG	TGGGCGAGCA	TCCCCTACGG	CCGTCCGCTG	CGCAACCAGA	CGTTCCACGT
	8821	GCTCGATGAG	GCGCTCGAAC	CGCGCCCGGT	CTGGGTTCCG	GGGCAACTCT	ACATTGGCGG
35	8881	GGTCGGACTG	GCACTGGGCT	ACTGGCCGGA	TGAAGAGAAG	ACGCGCAACA	CGTTCTCTCT
	8941	GCACCCCGAG	ACCGGGGAGC	GCCTCTACAA	GACCGGCGAT	CTGGGCGGCT	ACCTGCCCCG
	9001	TGGAACATC	GAGTTCATGG	GGCGGGAGGA	CAACCAAATC	AAGCTTCGCG	GATACCGCGT
	9061	TGAGCTCGGG	GAAATCGAGG	AAACGCTCAA	GTGCGATCCG	AACGTACGCG	ACGCGGTGAT
	9121	TGTGCCCGTC	GGGAACGACG	CGGCGAACAA	GCTCCTTCTA	GCCTATGTGG	TCCCAGGAAGG
40	9181	CACACGGA	CGCGCTGCCG	AGCAGGACGC	GAGCCTCAAG	ACCGAGCGGG	TCGACGCGAG
	9241	AGCACACGCC	GCCAAAGCGG	ACGGATTGAG	CGACGGCGAG	AGGGTGCAGT	TGAGCTCGC
	9301	TCGACACGGA	CTCCGGAGGG	ATCTGGACGG	AAAGCCCGTC	GTCGATCTGA	CCGGGCTGGT
	9361	TCCGCGGGAG	GCGGGGCTGG	ACGTCTACGC	GCGTCGCCGT	AGCGTCCGAA	CGTTCTCTCG
	9421	GGCCCCGATT	CCATTTGTTG	AATTCGGCCG	ATTCTGAGC	TGCCTGAGCA	GCGTGGAGCC
45	9481	CGACGGCGCG	GCCCTTCCCA	AATTCGGTTA	TCCATCGGCT	GGCAGCACGT	ACCCGGTGCA
	9541	AACCTACGCG	TACGCCAAAT	CCGGCCCGAT	CGAGGGCGTG	GACGAGGGCT	TCTATTATTA
	9601	CCACCCGTTT	GAGCACCGTT	TGCTGAAGGT	CTCCGATCAC	GGGATCGAGC	GCGGAGCGCA
	9661	CGTTCGCGAA	AACTTCGACG	TGTTTCGATGA	AGCGGCGTTC	GGCCTCCTGT	TCGTGGGCAG
	9721	GATCGATGCC	ATCGAGTCGC	TGTATGGATC	GTTGTACGGA	GAATTCTGCC	TGCTGGAGGC
50	9781	CGGATATATG	GCGCAGCTCC	TGATGGAGCA	GGCGCCTTCC	TGCAACATCG	GCGTCTGTCC
	9841	GGTGGGTCAA	TTCGATTTTG	AACAGGTTCC	GCCGGTTCTC	GACCTGCGGC	ATTCCGACGT
	9901	TTACGTGCAC	GGCATGCTGG	GCGGGCGGGT	AGACCCGCGG	CAGTTCAGG	TCTGTACGCT
	9961	CGGTCAGGAT	TCCTCACCGA	GGCGCGCCAC	GACGCGCGGC	GCCCCCTCCG	GCCCGGATCA
55	10021	GCACTTCGCC	GATATCCTTC	GCGACTTCTT	GAGGACCAAA	CTACCCGAGT	ACATGGTGCC
	10081	TACAGTCTTC	GTGGAGCTCG	ATGCGTTGCC	GCTGACGTCC	AACGGCAAGG	TCGATCGTAA
	10141	GGCCCTGCGC	GAGCGGAAGG	ATACCTCGTC	GCCGCGGCAT	TCGGGGCACA	CGGCGCCACG
	10201	GGACGCCTTG	GAGGAGATCC	TCGTTGCGGT	CGTACGGGAG	GTGCTCGGGC	TGGAGGTGGT
	10261	TGGGCTCCAG	CAGAGCTTCG	TCGATCTTGG	TGCGACATCG	ATTCACATCG	TTCGATGAG
	10321	GAGTCTGTTG	CAGAAGAGGC	TGGATAGGGA	GATCGCCATC	ACCGAGTTGT	TCCGATACCC
	10381	GAACCTCGGC	TCGCTGGCGT	CCGGTTTGCG	CCGAGACTCG	AAAGATCTAG	AGCAGCGGCC

10441 GAACATGCAG GACCGAGTGG AGGCTCGGCG CAAGGGCAGG AGACGTAGCT AAGAGCGCCC  
10501 AACAAAACCA GGCCGAGCGG GCCAATGAAC CGCAAGCCCG CCTGCGTCAC CCTGGGACTC  
10561 ATCTGATCTG ATCGCGGGTA CGCGTCGCGG GTGTGCGCGT TGAGCCGTGT TGCTCGAACG  
5 10621 CTGAGGAACG GTGAGTCTAT GGAAGAACAA GAGTCTCTCCG CTATCGCAGT CATCGGCATG  
10681 TCGGGCCGTT TTCCGGGGGC GCGGGATCTG GACGAATTCT GGAGGAACCT TCGAGACGGC  
10741 ACGGAGGCCG TGCAGCGCTT CTCCGAGCAG GAGCTCGCGG CGTCCGGAGT CGACCCAGCG  
10801 CTGGTGCTGG ACCCGAATA CGTCCGGGCG GGCAGCGTGC TGGAAGATGT CGACCCGTTT  
10861 GACGCTGCTT TCTTCGGCAT CAGCCCGCGC GAGGCAGAGC TCATGGATCC GCAGCACCGC  
10921 ATCTTCATGG AATGCGCCTG GGAGGCGCTG GAGAACGCCG GATACGACCC GACAGCCTAC  
10 10981 GAGGGCTCTA TCGGCGTGTA CGCCGGGCGC AACATGAGCT CGTACTTGAC GTCGAACCTC  
11041 CACGAGCACC CAGCGATGAT CCGGTGGCCC GGCTGGTTTC AGACGTTGAT CGGCAACGAC  
11101 AAGGATTACC TCGCGACCCA CGTCTCTTAC AGGCTGAATC TGAGAGGGCC GAGCATCTCC  
11161 GTTCAAACCTG CCTGCTCTAC CTCGCTCGTG GCGGTTCACT TGGCGTGAT GAGCCTCTTG  
11221 GACCGCGAGT GCGACATGGC GCTGGCCGGC GGGATTACCG TCCGGATCCC CCATCGAGCC  
15 11281 GGCTATGTAT ATGCTGAGGG GGGCATCTTC TCTCCGACG GCCATTGCCG GGCCTTCGAC  
11341 GCCAAGGCGA ACGGCACGAT CATGGGCAAC GGCTGCGGGG TTGTCTCTCT GAAGCCGCTG  
11401 GACCGGGGCGC TCTCCGATGG TGATCCCGTC CGCGCGGTCA TCCTTGGGTC TGCCACAAAC  
11461 AACGACGGAG CGAGGAAGAT CCGGTTCACT GCGCCAGTG AGGTGGGCCA GGCGCAAGCG  
20 11521 ATCATGGAGG CGCTGGCGCT GGCAGGGGTC GAGGCCCCGT CCATCCAATA CATCGAGACC  
11581 CACGGGACCG GCACGCTGCT CGGAGACGCC ATCGAGACGG CCGCGTTGCG GCGGTTGTTT  
11641 GATCGCGACG CTTTCGACCCG GAGGTCTTGC GCGATCGGCT CCGTGAAGAC CGGCATCGGA  
11701 CACCTCGAAT CGGCGGCTGG CATCGCCGGT TTGATCAAGA CCGTCTTGGC GCTGGAGCAC  
11761 CCGCAGCTGC CGCCAGCCT GAACTTCGAG TCTCTAACC CATCGATCGA TTTCCGCGAGC  
25 11821 AGCCCGTTCT ACGTCAATAC CTCTCTTAAG GATTGGAATA CCGGCTCGAC TCCGCGGCGG  
11881 GCCGCGTCA GCTCGTTCCG GATCGGCGGC ACCAACGCCC ATGTCGTGCT GGAGCAAGCA  
11941 CCCGCGCGCA AGCTTCCAGC CGCGCGCCG GCGCGCTCTG CCGAGCTCTT CGTCGTCTCG  
12001 GCCAAGAGCG CAGCGGCGCT GGATGCCGCG GCGGCACGGC TACGAGATCA TCTGCAGGCG  
12061 CACCAGGGGC TTTCGTTGGG CGACGTCGCG TTCAGCCTGG CGACGACCGC CAGTCCCATG  
30 12121 GAGCACCGGC TCGCGATGGC GGCACCGTCG CGCAGGCGT TGCGAGAGGG GCTCGACGCA  
12181 GCGGCGCGAG GCCAGACCCC GCGGGGCGCC GTGCGTGCGC GCTGCTCCCC AGGCAACGTG  
12241 CCGAAGGTGG TCTTCGTCTT TCCCGGCCAG GGCTCTCAGT GGGTCGGTAT GGGCCGTGAG  
12301 CTCCTGGCTG AGGAACCCGT CTTCCACGCG GCGCTTTCGG CGTGCGACCG GGCCATCCAG  
12361 GCGCAAGCTG GTTGGTCTGCT GCTCGCCGAG CTCGCCGCCG ACGAAGGGTC GTCCAGATC  
35 12421 GAGCGCATCG ACCTGGTGCA GCGGTGCTG TTCGCGCTCG CCGTGGCATT TGCGGCGCTG  
12481 TGGCGGTCTG GGGGTGTCGG GCGCGACGTC GTGATCGGCC ACAGCATGGG CGAGGTAGCC  
12541 GCGCGCATG TGGCCGGGGC GCTGTGCTC GAGGATGCGG TGCGGATCAT CTGCGGCGC  
12601 AGCCGGCTGC TCCGGCGCAT CAGCGGTCAG GCGGAGATGG CCGTGACCGA GCTGTCTGTG  
12661 GCGGAGGCGG AGGCAGCGCT CCGAGGCTAC GAGGATCGGG TGAGCGTGGC CGTGAGCAAC  
40 12721 AGCCCGCGCT CGACGGTGCT CTCGGGCGAG CCGGCAGCGA TCGGCGAGGT GCTGTCTGTC  
12781 CTGAACGCGA AGGGGTGTT CTGCCGTCGG GTGAAGGTGG ATGTCGCCAG CCACAGCCCG  
12841 CAGGTCGACC CGCTGCGCGA GGACCTCTTG GCAGCGCTGG GCGGGCTCCG GCCGCGTGCG  
12901 GCTGCGGTGC CGATGCGCTC GACGGTGACG GCGGCCATGG TAGCGGGCCC GGAGCTCGGA  
12961 GCGAATTACT GGATGAACAA TCTCAGGCAG CCTGTGCGCT TCGCCGAGGT AGTCCAGGCG  
45 13021 CAGCTCCAAG GCGGCCACGG TCTGTTCGTG GAGATGAGCC CGCATCCGAT CCTAACGACT  
13081 TCGGTCGAGG AGATGCGCGG CGCGGCCAG CCGGCGGGCG CAGCGGTGGG CTCGTGCGG  
13141 CGAGGGCAGG ACGAGCGCCC GCGATGCTG GAGGCGCTGG GCGCGCTGTG GCGCAGGGG  
13201 TACCCTGTAC CCTGGGGGCG GCTGTTTCCC GCGGGGGGGC GGCGGGTACC GCTGCCGACC  
13261 TATCCCTGGC AGCGCGAGCG GTACTGGATC GAAGCGCCGG CCAAGAGCGC CGCGGGCGAT  
50 13321 CGCCGCGGCG TGCGTGCGGG CGGTACCCCG CTCCTCGGTG AAATGCAGAC CCTATCAACC  
13381 CAGACGAGCA CGCGGCTGTG GGAGACGACG CTGGATCTCA AGCGGCTGCC GTGGCTCGGC  
13441 GACCACGGG TGCAGGGAGC GGTCTGTGTT CCGGGCGCGG CGTACCTGGA GATGGCGATT  
13501 TCGTCGGGGG CCGAGGCTTT GGGCATGCGC CCATTGCGA TAACCGACGT GGTGCTCGCC  
13561 GAGGCGCTGG CCTTCGCGGG CGACGCGGCG GTGTTGGTCC AGGTGGTGAG GACGGAGCAG  
55 13621 CCGTCGGGAC GGCTGCAGTT CCAGATCGCG AGCCGGGCGC GGGGCGCTGG CCACGCGTCC  
13681 TTCCGGGTCC ACGCTCGCGG CGCGTTGCTC CGAGTGAGC GCACCGAGGT CCCGGCTGGG  
13741 CTTACGCTTT CCGCGTGCG CGCACGGCTC CAGGCCAGCA TGCCCGCCGC GGCCACCTAC  
13801 GCGGAGCTGA CCGAGATGGG GCTGCACTAC GGCCCTGCCT TCCAGGGGAT TGCTGAGCTA  
13861 TGGCGCGGTG AGGGCGAGGC GTCGACAGG GTACGCTGC CCGACGCGGC CCGCTCGGCA  
13921 GCGGAGTATC GGTGTCATCC TGCGCTGCTG GACGCGTGCT TCCAGGTCTG CCGCAGCCTC

13981 TTCGCCGGCG GTGGCGAGGC GACGCCGTGG GTGCCCGTGG AAGTGGGCTC GCTGCGGCTC  
14041 TTGCAGCGGC CTTCCGGGGA GCTGTGGTGC CATGCGCGCG TCGTGAACCA CGGGCGCCAA  
14101 ACCCCCGATC GGCAGGGCGC CGACTTTTGG GTGGTCGACA GCTCGGGTGC AGTGGTCGCC  
5 14161 GAAGTCAGCG GGCTCGTGGC GCAGCGGCTT CCGGGAGGGG TCGCCCGGCG CGAAGAAGAC  
14221 GATTGGTTCC TGGAGCTCGA GTGGGAACCC GCAGCGGTCC GCACAGCCAA GGTCACGCG  
14281 GGCCGGTGGC TGCTCCTCGG CGGCGGCGGT GGGCTCGGCG CCGCGTTGCG CTCGATGCTG  
14341 GAGGCCGGCG GCCATGCCGT CGTCCATGCG GCAGAGAGCA ACACGAGCGC TGCCGGCGTA  
14401 CGCGCGCTCC TGGCAAAGGC CTTTGACGGC CAGGCTCCGA CGGCGGTGGT GCACCTCGGC  
10 14461 AGCCTCGATG GGGGTGGCGA GCTCGACCCA GGGCTCGGGG CGCAAGGCGC ATTGGACGCG  
14521 CCCCGGAGCG CCGACGTCAG TCCCGATGCC CTCGATCCGG CGCTGGTACG TGGCTGTGAC  
14581 AGCGTGCTCT GGACCGTGCA GGCCCTGGCC GGCATGGGCT TTCGAGACGC CCGCGATTG  
14641 TGGCTTCTGA CCCGCGGCGC ACAGGCCGTC GGCAGCGGCG ACGTCTCCGT GACACAGGCA  
14701 CCGTCTGTGG GGCTGGGCGG CGTCATCGGC ATGGAGCAGC CGGATCTGCG CTGCGCTCGG  
14761 GTCGACCTCG ATCCGACCCG GCGCGATGGG GAGCTCGGTG CCCTGCTGGC CGAGCTGCTG  
15 14821 GCGGACGACG CCGAAGCGGA AGTCGCGTTG CCGGGTGGCG AGCGATGCGT CGCTCGGATC  
14881 GTCCGCCGGC AGCCCGAGAC CCGCCCCCGG GGGAGGATCG AGAGCTGCGT TCCGACCGAC  
14941 GTCACCATCC GCGCGGACAG CACCTACCTT GTGACCGGCG GTCTGGGTGG GCTCGGTCTG  
15001 AGCGTGGCGG GATGGCTGGC CGAGCGCGGC GCTGGTCACC TGGTGCTGGT GGGCCGCTCC  
15061 GCGCGGCGCA GCGTGGAGCA ACGGGCGAGC GTCGCGGCGC TCGAGGCCCG CGGCGCGCGC  
20 15121 GTCACCGTGG CGAAGGCAGA TGTCGCCGAT CCGGCGCAGC TCGAGCGGAT CCTCCGCGAG  
15181 GTTACCACGT CCGGGATGCC GCTGCGGGGC GTCGTCCATG CCGCCGGCAT CTTGGACGAC  
15241 GGGCTGCTGA TGCAGCAGAC TCCCGCGCGG TTTCTGTAAG TGATGGCGCC CAAGGTCCAG  
15301 GGGGCTTTCG ACCTGCACGC GTTGACGCGC GAAGCGCCGC TTTCTTCTT CGTGCTGTAC  
15361 GCTTCGGGAG TAGGGCTCTT GGGCTCGCGG GGCCAGGGCA ACTACGCCGC GGCCAACACG  
25 15421 TTCTTCGACG CTCTGGCGCA CCACCGGAGG GCGCAGGGGC TGCCAGCGTT GAGCGTCGAC  
15481 TGGGGCCTGT TCGCGGAGGT GGGCATGGCG GCGCGCAGG AAGATCGCGG CGCGCGGCTG  
15541 GTCTCCCGCG GAATGCGGAG CCTACCCCC GACGAGGGGC TGTCCGCTCT GGCACGGCTG  
15601 CTCGAAAGCG GCCGCGTGCA GGTGGGGGTG ATGCCGGTGA ACCCGCGGCT GTGGGTGGAG  
15661 CTCTACCCCG CGGCGGCGTC TTCGCGAATG TTGTCGCGCC TGGTGACGGC GCATCGCGCG  
30 15721 AGCGCCGGCG GGCCAGCCGG GACGCGGAGC CTGCTCCGCC GCCTCGCTGC TGCCGAGCCG  
15781 AGCGCGCGGA GCGGGCTCCT GCGGGCGCTC TCCGCGCGC AGATCTCGCA GGTGCTGCGC  
15841 CTCCCGGAGG GCAAGATCGA GGTGGACGCC CCGCTCACGA GCCTGGGCAT GAACTCGCTG  
15901 ATGGGGCTCG AGCTGCGCAA CCGCATCGAG GCCATGCTGG GCATCACCGT ACCGGCAACG  
15961 CTGTTGTGGA CCTATCCAC GGTGGCGGCG CTGAGCGGGC ATCTGGCGCG GGAGGCATGC  
35 16021 GAAGCCGCTC CTGTGGAGTC ACCGCACACC ACCGCCGATT CTGCTGTCTGA GATCGAGGAG  
16081 ATGTCCGAGG ACGATCTGAC CAGTTGATC GCAGCAAAAT TCAAGGCGCT TACATGACTA  
16141 CTCGCGGTCC TACGGCACAG CAGAATCCGC TGAACAAGC GGCCATCATC ATTCAGCGGC  
16201 TGGAGGAGCG GCTCGCTGGG CTCGCACAGG CCGAGCTGGA ACGGACCGAG CCGATCGCCA  
16261 TCGTCGGTAT CCGCTGCCGC TTCCCTGGCG GTGCGGACGC TCCGGAAGCG TTTTGGGAGC  
40 16321 TGCTCGACGC GGAGCGCGAC GCGGTCCAGC CGCTCGACAG GCGCTGGGCG CTGGTAGGTG  
16381 TCGTCCCGT CGAGGCCGTG CCGCACTGGG CCGGGCTGCT CACCGAGCCG ATAGATTGCT  
16441 TCGATGCTGC GTTCTTCGGC ATCTCGCCTC GGGAGGCGCG ATCGCTCGAC CCGCAGCATC  
16501 GTCTGTTGCT GGAGGTGCTG TGGGAGGGGC TCGAGGACGC CCGTATCCCG CCCCCTCCA  
16561 TCGACGGGAG CCGCACCGGT GTGTTCTGTC GCGCTTTCAC GCGGACTAC GCGCGCACGG  
45 16621 TCGCTCGGTT GCGCGCGGAG GAGCGAGACG CGTACAGCGC CACCGGCAAC ATGCTCAGCA  
16681 TCGCCGCGCG ACGGCTGTCT TACACGCTGG GGCTGCAGG ACCTTGCCCTG ACCGTCGACA  
16741 CGCGGTGCTC GTCATCGCTG GTGGCGATT ACCTCGCCTG CCGCAGCCTG CGCGCAGGAG  
16801 AGAGCGATCT CGCGTTGGCG GGAGGGGTCA GCACGCTCCT CTCCCCGAC ATGATGGAAG  
16861 CCGCGGCGCG CACGCAAGCG CTGTCGCCCC ATGGTCGTTG CCGGACCTTC GATGCTTCGG  
50 16921 CCAACGGGTT CGTCCGTGGC GAGGGCTGTG GCCTGGTCGT CCTCAAACGG CTCTCCGACG  
16981 CGCAACGGGA TGGCGACCGC ATCTGGGCGC TGATCCGGGG CTCGGCCATC AACCATGATG  
17041 GCGGTCGAC CCGGTTGACC GCGCCCAACG TGCTGGCTCA GGAGACGGTC TTGCGCGAGG  
17101 CGCTCGGAG CGCCCACGTC GAAGCTGGGG CCGTCGATTA CGTCGAGACC CACGGAACAG  
17161 GGACCTCGCT GGGCGATCCC ATCGAGGTCG AGGCGCTGCG GCGGACGGTG GGGCCGGCGC  
55 17221 GCTCCGACGG CACACGCTGC GTGCTGGGCG CCGTGAAGAC CAACATCGG CATCTCGAGG  
17281 CCGCGGAGG CGTAGCGGGC CTGATCAAGG CAGCGCTTTC GCTGACGCAC GAGCGCATCC  
17341 CGAGAAACCT CAACTTCCGC ACGCTCAATC CCGGATCCG GCTCGAGGGC AGCGCGCTCG  
17401 CGTTGGCGAC CGAGCCGGTG CCGTGGCCGC GCACGGACCG TCCGCGCTTC GCGGGGGTGA  
17461 GCTCGTTCCG GATGAGCGGA ACGAACGCGC ATGTGGTGCT GGAAGAGGCG CCGCGGTTG

17521 AGCTGTGGCC TGCCGCGCCG GAGCGCTCGG CGGAGCTTTT GGTGCTGTCT GGCAAGAGCG  
17581 AGGGGGCGCT CGACGCGCAG GCGGCGCGGC TCGCGAGCA CCTGGACATG CACCCGAGAGC  
17641 TCGGGCTCGG GGACGTGGCG TTCAGCCTGG CGACGACGCG CAGCGCGATG ACCCACCAGG  
17701 TCGCGGTGGC GGTGACGTCG CGCGAGGGGC TGCTGGCGGC GCTTTCGGCC GTGGCGCAGG  
5 17761 GGCAGACGCC GCGGGGGGCG GCGCGCTGCA TCGCGAGCTC CTCGCGCGGC AAGCTGGCGT  
17821 TGCTGTTTAC CGGACAGGGC GCGCAGACGC CGGGCATGGG CCGGGGGCTC TCGCGCGCGT  
17881 GGCCAGCGTT CCGGGAGGCG TTCCAGCCGT GCGTGACGCT GTTCGACCGG GAGCTGGACC  
17941 GCGCGCTGCG CGAGGTGATG TGGGCGGAGG CGGGGAGCGC CGAGTCGTTG TCGCTGGACC  
10 18001 AGACGGCGTT CACCCAGCCC GCGCTCTTCG CGGTGGAGTA CGCGCTGACG GCGCTGTGGC  
18061 GGTGCTGGGG CGTAGAGCCG GAGCTCCTGG TTGGGCATAG CATCGGGGAG CTGGTGGCGG  
18121 CGTGCCTGGC GGGGGTGTTC TCGCTGGAAG ATGGGGTGAG GCTCGTGGCG GCGCGCGGGC  
18181 GGCTGATGCA GGGGCTCTCG CGGGCGCGCG CGATGGTGTC GCTCGGAGCG CCGGAGGCGG  
18241 AGGTGGCCGC GCGGCTGGCG CCGCAGCGCG CTGGGTGTC GATCGCGCGG GTCAATGGGC  
18301 CGGAGCAGGT GGTGATCGCG GCGGTGGAGC AAGCGGTGCA GGCGATCGCG GCGGGGTTCG  
15 18361 CGGCGCGCGG CGTGCGCACC AAGCGGCTGC ATGTCTCGCA CGCGTTCCAC TCGCGCTGA  
18421 TGGAACCGAT GCTGGAGGAG TTCGGGCGGG TGGCGGCGTC GGTGACGTAC CCGCGGCCAA  
18481 GCGTTTCGCT GGTGAGCAAC CTGAGCGGGA AGGTGGTCAC GGACGAGCTG AGCGCGCCG  
18541 GCTACTGGGT GCGGCACGTG CCGGAGCGCG TCGGCTTCGC GGACGGGGTG AAGCGCTGC  
20 18601 ACGAAGCCGG CGCGGGCAGG TTCTCGAAG TGGCCCCGAA GCCGACGCTG CTCGGCCTGT  
18661 TGCCAGCTTG CCTGCCGGAG GCGGAGCCGA CGTTGCTGGC GTCGTTGCGG GCGGGCGCG  
18721 AGGAGCTGCG GGGGGTGTCT GAGGCGCTGG GCAGGCTGTG GGCCGCTGGC GGCTCGGTCA  
18781 GCTGGCCGGG CGTCTTCCCC ACGGCTGGGC GCGGGGTGCC GCTGCCGACC TATCCGTGGC  
18841 AGCGGCAGCG GTACTGGATC GAGGCGCCGG CCGAAGGGCT CGGAGCCACG GCGCGCGATG  
25 18901 CGCTGGCGCA GTGGTTCTAC CGGGTGGACT GGCCCGAGAT GCCTCGCTCA TCCGTGGATT  
18961 CGCGGCGAGC CCGGTCCGGC GGTGCTGGT TGCTGGCCGA CCGGGGTGGA GTCGGGGAGG  
19021 CGGCCGCGGC GCGGCTTTTCG TCGCAGGGAT GTTCGTGCGC CGTGCTCCAT GCGCCGCTG  
19081 AGGCCTCCGC GGTGCGCCAG CAGGTGACCC AGGCCTCGG TGGCCGCAAC GACTGGCAGG  
19141 GGGTGCTGTA CCTGTGGGGT CTGGACGCCG TCGTGGAGGC GGGGCGATCG GCCGAAGAGG  
30 19201 TCGGCAAGT CACCCATCTT GCCACGGCG CGGTGCTCGC GCTGATTACG GCGGTGGGCA  
19261 CCGGGCCGCG CTCACCCCG CTCTGGATCG TGACCCGAGG GGCTGACAG GTGGGCGGGC  
19321 AGCCTGACGC TGCCCCCTGT CAGGCGGCGC TGTGGGGTAT GGGCCGGGTG GCGCGCTGG  
19381 AGCATCCCG CTCTGGGGC GGGCTCGTGG ACCTGGATCC GGAGGAGAGC CCGACGGAGG  
19441 TCGAGGCCCT GGTGGCCGAG CTGCTTTCGC CGGACGCCGA GGATCAGCTG GCATTCCGCC  
35 19501 AGGGCGCGCG GCGCGCAGCG CCGCTCGTGG CCGCCCCACC GGAGGGAAC GCAGCGCCGG  
19561 TGTCGCTGTC TGCGGAGGGG AGTTACTTGG TGACGGGTGG GCTGGGCGCC CTTGGCCTCC  
19621 TCCTTGCGCG GTGGTTGGTG GAGCGCGGG CGGGCACCT TGTGCTGATC AGCCGGCAGC  
19681 GATTGCCCCA CCGCGAGGAA TGGGGCCGAG ATCAGCCGCC AGAGGTGCGC GCGCGATTG  
19741 CCGCGATCGA GCGCTGGAG GCGCAGGGCG CGCGGGTCAC CGTGGCGGCG GTCGACGTGG  
40 19801 CCGATGCCGA AGGCATGGCG GCGCTCTTGG CCGCGCTCGA GCGCGCGCTG CCGGGGGTGG  
19861 TGCACGCCG GGGTCTGCTC GACGACGGGG TGCTGGCCCA CCAGGACGCC GGTGCGCTCG  
19921 CCGGGGTGTT GCGCCCCAAG GTGGAGGGGG CATGGGTGCT GCACACCCTT ACCCGCGAGC  
19981 AGCCGCTGGA CCTCTTCGTA CTGTTTTCTT CCGCGTCCGG CGTCTTCGGC TCGATCGGCC  
20041 AGGGCAGCTA CCGGCGAGGC AATGCCTTTT TGGACGCGCT GGCGGACCTC CGTCGAACGC  
45 20101 AGGGGCTCGC CGCCTGAGC ATCGCCTGGG GCCTGTGGGC GGAGGGGGGG ATGGGCTCGC  
20161 AGGCGCAGCG CCGGGAACAT GAGGCATCGG GAATCTGGGC GATGCCGAGC AGTCGTGCCC  
20221 TGGCGGCGAT GGAATGGCTG CTCGGTACGC GCGCGACGCA GCGCGTGGTC ATCCAGATGG  
20281 ATTGGGCCCA TGCGGAGCG GCTCCGCGG ACGCGAGCCG AGGCCGCTTC TGGGATCGGC  
20341 TGGTAACTGT CACGAAAGCG GCCTCTCTCT CCGCGGTGCC AGCTGTAGAG CGCTGGCGCA  
50 20401 ACGGCTCTGT TGTGGAGACC CGCTCGGCG TCTACGAGCT TGTGCGCGGC GTGGTCCCG  
20461 GGGTGATGGG CTTTACCGAC CAAGGCACGC TCGACGTGCG ACGAGGCTTC GCCGAGCAGG  
20521 GCCTCGACTC CCTGATGGCT GTGGAGATCC GCAAACGGCT TCAGGGTGAG CTGGGTATGC  
20581 CGCTGTCCGC GACGCTGGCG TTCGACCATC CGACCGTGGA GCGGCTGGTG GAATACTTGC  
20641 TGAGCCAGGC GCTGGAGCTG CAGGACCGCA CCGACGTGCG AAGCGTTCGG TTGCCGGCGA  
55 20701 CAGAGGACCC GATCGCCATC GTGGGTGCG CCGGCTGCTT CCGGGGCGGG GTCCAGGACC  
20761 TGGAGTCTTA CTGGCAGCTG TTGACCGAGG GCGTGGTGGT CAGCACCAG GTGCCGGCCG  
20821 ACCGGTGGAA TGGGGCAGAC GGGCGCGGCC CCGGCTCGGG AGAGGCTCCG AGACAGACCT  
20881 ACGTGCCAG GGGTGGCTTT CTGCGCGAGG TGGAGACGTT CGATGCGGCG TTCTTCCACA  
20941 TCTCGCCTCG GGAGGCGATG AGCCTGGACC CGCAACAGCG GCTGCTGCTG GAAGTGAGCT  
21001 GGGAGGCGAT CGAGCGCGCG GCGCAGGACC CGTCGGCGCT GCGCGAGAGC CCCACGGGCG

21061 TGTTCTGTGGG CGCGGGCCCC AACGAATATG CCGAGCGGGT GCAGGACCTC GCCGATGAGG  
21121 CGGCGGGGCT CTACAGCGGC ACCGGCAACA TGCTCAGCGT TGCGGCGGGA CGGCTGTCAT  
21181 TTTTCCTGGG CCTGCACGGG CCGACCTTGG CTGTGGATAC GGCGTGCTCC TCGTCGCTCG  
5 21241 TGGCGCTGCA CCTCGGCTGC CAGAGCTTGC GACGGGGCGA GTGCGACCAA GCCCTGGTTG  
21301 GCGGGGTCAA CATGCTGCTC TCGCCGAAGA CCTTCGCGCT GCTCTCACGG ATGCACGCGC  
21361 TTTGCCCCGG CGGGCGGTGC AAGACGTTCT CGGCCGACGC GGACGGCTAC GCGCGGGCCG  
21421 AGGGCTGCGC CGTGGTGGTG CTCAAGCGGC TCTCCGACGC GCAGCGCGAC CGCGACCCCA  
21481 TCCTGGCGGT GATCCGGGGT ACGGCGATCA ATCATGATGG CCCGAGCAGC GGGCTGACAG  
10 21541 TGCCCAGCGG CCCTGCCCCAG GAGCGCTGT TACGCCAGGC GCTGGCGCAC GCAGGGGTGG  
21601 TTCCGGCCGA CGTCGATTTT GTGGAATGCC ACGGGACCGG GACGGCGCTG GCGACCCCA  
21661 TCGAGGTGCG GCGGCTGAGC GACGTGTACG GGCAAGCCCG CCCTGCGGAC CGACCGCTGA  
21721 TCCTGGGAGC CGCCAAGGCC AACCTTGGGC ACATGGAGCC CGCGGCGGGC CTGGCCGGCT  
21781 TGCTCAAGGC GGTGCTCGCG CTGGGGCAAG AGCAAATACC AGCCCAGCCG GAGCTGGCCG  
15 21841 AGCTCAACCC GCTCTTGCCG TGGGAGGCGC TGCCGGTGGC GGTGGCCCGC GCAGCGGTGC  
21901 CGTGGCCCGC CACGGACCGT CCGCGCTTCG CGGGGGTGAG CTCGTTCGGG ATGAGCGGAA  
21961 CGAACGCGCA TGTGGTGCTG GAAGAGGCGC CGGCGGTGGA GCTGTGGCCT GCCGCGCCCG  
22021 AGCGCTCGGC GGAGCTTTTG GTGTGTTCGG GCAAGAGCGA GGGGGCGCTC GACGCGCAGG  
22081 CGGCGCGGCT GCGCGAGCAC CTGGACATGC ACCCGGAGCT CGGGCTCGGG GAGCTGGCGT  
20 22141 TCAGCCTGGC GACGACGCGC AGCGCGATGA ACCACCGGCT CGCGGTGGCG GTGACGTCGC  
22201 GCGAGGGGCT GCTGGCGGCG CTTTCGGCCG TGGCGCAGGG GCAGACGCCG CCGGGGGCGG  
22261 CGCGCTGCAT CCGGAGCTCG TCGCGCGGCA AGCTGGCGTT CCTGTTCACC GGACAGGGCG  
22321 CGCAGACGCC GGGCATGGGC CGGGGGCTTT GCGCGGCGTG GCCAGCGTTC CGAGAGGCGT  
22381 TCGACCGGTG CGTGGCGCTG TTCGACCAGG AGCTGGACCG CCCGCTGTGC GAGGTGATGT  
22441 GGGCGGAGCC GGGGAGCGCC GAGTCTGTGT TGCTCGACCA GACGGCGTTC ACCCAGCCCG  
25 22501 CGCTCTTCAC GGTGGAGTAC GCGCTGACGG CGCTGTGGCG GTCGTGGGCG GTGCGTGGCG  
22561 AGCTGGTGGC TGGGCATAGC GCCGGGGAGC TGGTGGCGGC GTGCGTGGCG GGGGTGTTCT  
22621 CGCTGGAAGA TGGGGTGAGG CTCGTGGCGG CGCGCGGGCG GCTGATGCAG GGGCTCTCGG  
22681 CGGGCGGCGC GATGGTGTCG CTCGGAGCGC CGGAGGCGGA GGTGGCCCGC GCGGTGGCGC  
22741 CGCACGCGGC GTGGGTGTCT ATCGCGCGCG TCAATGGGCC GGAGCAGGTG GTGATCGCGG  
30 22801 GCGTGGAGCA AGCGGTGCAG GCGATCGCGG CGGGGTTTCG GCGCGCGGCG GTAGAGCCCG  
22861 AGCGGTGCA TGTCTCGCAC GCATCCCACT CGCCGCTGAT GGAACCGATG CTGGAGGAGT  
22921 TCGGGCGGGT GCGGCGGTCG GTGACGTACC GCGCGCCAAG CGTTTCGCTG GTGAGCAACC  
22981 TGAGCGGGAA GGTGGTCACG GACGAGCTGA GCGCGCCCGG CTACTGGGTG CCGCACGTGC  
35 23041 GGGAGGCGGT GCGCTTCGCG CACGGGGTGA AGGCGTGCA CGAAGCCGGC GCGGGGACGT  
23101 TCCTCGAAGT GGGCCCGAAG CCGACGCTGC TCGGCTGTT GCCAGCTTG CTGCCGGAGG  
23161 CGGAGCCGAC GCTGCTGGCG TCGTTGCGCG CCGGGCGCGA GGAGGCTGCG GGGGTGCTCG  
23221 AGGCGTGGG CAGGCTGTGG GCCGCCGGCG GCTCGGTCAG CTGGCCGGGC GTCTTCCCCA  
23281 CGGCTGGGCG GCGGGTGCCG CTGCCGACCT ATCCGTGGCA GCGGCAGCGG TACTGGCCCG  
23341 ACATCGAGCC TGACAGCCGT CGCCACGCA CCGCGGATCC GACCCAAGGC TGGTTCTATC  
40 23401 GCGTGGACTG GCCGAGATA CCTCGAGCC GCGCGAGATC TCCAGAAATC AGAGGAGGCG AGCCGCGGGA  
23461 GCTGGCTGGT ATTGGCGGAT AAGGGTGGAG TCGGCGAGGC GGTGCTGCGA GCGCTGTGCA  
23521 CACGTGGACT TCCATGCGTC GTGCTCCATG CGCCGGCAGA GACATCCGCG ACCGCTGAGC  
23581 TGGTGACCGA GGCTGCCGCG GGTGCAAGCG ATTGGCAGGT AGTGCTCTAC CTGTGGGGTC  
45 23641 TGGACGCCGT CGTCGGCGCG GAGGCGTCGA TCGATGAGAT CGGCGACGCG ACCCGTCTGT  
23701 CTACCGCGCC GGTGCTCGGC TTGGCTCGGT TTCTGAGCAC CGTGTCTTGT TCGCCCCGAC  
23761 TCTGGGTGCT GACCCGGGGG GCATGCATCG TTGGCGACGA GCCTGCGATC GCCCTTGTG  
23821 AGGCGGCGTT ATGGGGCATG GGCCGGGTGG CGCCCGGCTG GATCCCGGG GACGCGGCGA  
23881 GGCTCGTGGA CCTGGATCCC CGAGCGAGCC CGCCCAAGC CAGCCCGATC GACGGCGAGA  
50 23941 TGCTCGTCAC CGAGCTATTG TCGCAGGAGA CCGAGGACCA GCTCGCCTTC CGCCATGGGC  
24001 GCCGGCACGC GGCACGGCTG GTGGCCGCCC CGCCACGGGG GGAAGCGGCA CCGGCGTCGC  
24061 TGTCTGCGGA GGCGAGCTAC CTGGTGACGG GAGGCTCGG TGGGCTGGGC CTGATCGTGG  
24121 CCCAGTGGCT GGTGGAGCTG GGAGCGCGCG ACTTGGTGCT GACCAGCCGG CCGGGGTTGC  
24181 CCGACCGGCA GCGGTGGCGG GAGCAGCAGC CGCCTGAGAT CCGCGCGCGG ATCGCAGCGG  
24241 TCGAGGCGCT GGAGGCGCGG GGTGCACGGG TGACCGTGGC AGCGGTGGAC GTGGGTCACG  
55 24301 TCGAACCGAT GACAGCGCTG GTTTCGTGCG TCGAGCCCCC GCTGCGAGGG GTGGTGCACG  
24361 CCGCTGGCGT CAGCGTCATG CGTCCACTGG CCGGAGACGGA CGAGACCCTG CTCGAGTCGG  
24421 TGCTCCGTCC CAAGGTGGCC GGGAGCTGGC TGCTGCACCG GCTGCTGCAC GGCCGCGCTC  
24481 TCGACCTGTT CGTGTGTTT TCGTCGGCG CAGCGGTGTG GGGTAGCCAT AGCCAGGGTG  
24541 CGTACGCGGC GGCCAACGCT TTCCTCGACG GGCTCGCGCA TCTTCGGCGT TCGCAATCGC



24601 TGCCTGCGTT GAGCGTCGCG TGGGGTCTGT GGGCCGAGGG AGGCATGGCG GACGCGGAGG  
24661 CTCATGCACG TCTGAGCGAC ATCGGGGTTC TGCCCATGTC GACGTGCGCA GCGTTGTCGG  
24721 CGCTCCAGCG CCTGGTGGAG ACCGGCGCGG CTCAGCGCAC GGTGACCCGG ATGGACTGGG  
5 24781 CGCGCTTCGC GCCGGTGTAC ACCGCTCGAG GCGTTCGCAA CCTGCTTTTC GCGCTGGTCG  
24841 CAGGGCGCGA CATCATCGCG CCTTCCCCTC CGGCGGCAGC AACC CGGAAC TGGCGTGGCC  
24901 TGTCCGTTCG GGAAGCCCGC ATGGCTCTGC ACGAGGTCGT CCATGGGGCC GTCGCTCGGG  
24961 TGCTGGGCTT CCTCGACCCG AGCGCGCTCG ATCCTGGGAT GGGGTTCAAT GAGCAGGGCC  
25021 TCGACTCGTT GATGGCGGTG GAGATCCGCA ACCTCCTTCA GGCTGAGCTG GACGTGCGGC  
25081 TTTGACGAC GCTGGCCTTT GATCATCCGA CCGTACAGCG GCTGGTGGAG CATCTGCTCG  
10 25141 TCGATGTACT GAAGCTGGAG GATCGCAGCG ACACCCAGCA TGTTCGGTCG TTGGCGTCAG  
25201 ACGAGCCCAT CGCCATCGTG GGAGCCGCGT GCGGCTTCCC GGGCGGGGTG GAGGACCTGG  
25261 AGTCTACTG GCAGCTGTTG TCCGAGGGCG TGGTGGTCAG CGCCGAGGTG CCGGCCGACC  
25321 GGTGGGATGC GCGGGAAGTG TACGACCTG ATCCGGAGAT CCCAGGCCGG ACTTACGTGA  
15 25381 CCAAAGGCGC CTTCTGCGC GATTTCGAGA GATTGGATGC GACCTTCTTC GCATCTCGC  
25441 CTCGCGAGGC GATGAGCCTC GACCCGACGC AGCGGTTGCT CCTGGAGGTA AGCTGGGAGG  
25501 CGCTCGAGAG CGCGGGTATC GCTCCGGATA CGCTGCGAGA TAGCCCCACC GGGGTGTTTC  
25561 TGGGTGCGGG GCCCAATGAG TACTACACGC AGCGGCTGCG AGGCTTCACC GACGGAGCGG  
25621 CAGGGCTGTA CGGCGGCACC GGAACATGC TCAGCGTTGC GGCTGGACGG CTGTCTGTTTT  
20 25681 TCCTGGGTCT GCACGGCCCG ACCTGCGCA TGGATACGGC GTGCTCGTCC TCCCTGGTCG  
25741 CGCTGCACCT CGCCTGCCAG AGCCTGCGAC TGGGCGAGTG CGATCAAGCG CTGGTTGGCG  
25801 GGGTCAACGT GCTGCTCGCG CCGGAGACCT TCGTGTGCT CTCACGGATG CGCGCGCTTT  
25861 CGCCCGACGG GCGGTGCAAG ACGTTCTCGG CCGACGCGGA CGGCTACGCG CGGGGCGAGG  
25921 GGTGCGCCGT GGTGGTGCTC AAGCGGTGCG GCGATGCGCA GCGCGCCGGC GACTCCATCC  
25981 TGGCGCTGAT CCGGGGAAGC CCGGTGTAAC ACACGCGCCC GAGCAGCGGG CTGACCGTGC  
25 26041 CCAACGGACC CGCCAGCAA GCATTGTGCG GCCAGGCGCT TTCGCAAGCA GGCCTGTCTC  
26101 CCGTTCGACGT TGATTTTGTG GAGTGTACAG GGACAGGGAC GCGCTGCGG GACCCGATCG  
26161 AGGTGCAGGC GCTGAGCGAG GTGTATGGTC CAGGGCGCTC CGAGGATCGA CCGCTGGTGC  
26221 TGGGGGCCGT CAAGGCCAAC GTCGCGCATC TGGAGGCGGC ATCCGGCTTG GCCAGCCTGC  
30 26281 TCAAGGCCGT GCTTGCCTG CGGCACGAG AGATCCCGGC CCAGCCGGAG CTGGGGGAGC  
26341 TCAACCCGCA CTTGCCGTGG AACACGCTGC CCGTGGCGGT GCCACGTAAG GCGGTGCCGT  
26401 GGGGCGCGCG CGCACGGCCG CGTGGGCGCG GCGTGAGCGC GTTCGGTTG AGCGGAACCA  
26461 ACGTGCATGT CGTGCTGGAG GAGGCACCGG AGGTGGAGCT GGTGCCCGCG GCGCCGCGCG  
26521 GACCGGTGGA GCTGGTTGTG CTATCGGCCA AGAGCGCGGC GCGCTGGAC GCCGCGCGCG  
35 26581 AACGGCTCTC GGCGCACCTG TCCGCGCACC CGGAGCTGAG CCTCGGCGAC GTGGCGTTCA  
26641 GCCTGGCGAC GACGCGCAG CCGATGGAG ACCGGCTCGC CATCGCGACG ACCTCGCGCG  
26701 AGGCCCTGCG AGGCGCGCTG GACGCGCGCG CGCAGCGCA GACGCCGAG GCGCGGTCG  
26761 GCGCAAGGC CGTGTCTCTA CGCGGTAAGT TGGCTTTTCT GTTCACCGGA CAGGGCGCGC  
26821 AAATGCCGGG CATGGGCCGT GGGCTGTACG AGGCGTGGCC AGCGTTCCGG GAGCGTTTCG  
40 26881 ACCGGTGGCT GGCGCTCTTC GATCGGGAGC TCGACAGCC TCTGCGCGAG GTGATGTGGG  
26941 CTGCGCCGGG CCTCGCTCAG GCGGCGCGGC TCGATCAGAC CGCGTACCG CAGCCGGCTC  
27001 TCTTTGCGCT TCGGCTGCCC TGGCTGCCC TGTGGCGTTC GTGGGGCGTG GAGCCGCACG  
27061 TACTCCTCGG TCATAGCATC GGCAGCTGG GTGCGCGCTG CGTGGCGGGG GTGTTCTCGC  
27121 TCGAAGACGC GGTGAGGTTG GTGGCCGCGC GCGGGCGGCT GATGCAGGC CTGCCCGCG  
45 27181 GCGGTGCCAT GGTGCGCCAT GCAGCGTCCG AGGCCGAGGT GGCCGCTCC GTGGCACCCC  
27241 ACGCCGCCAC GGTGTCGATC GCCCGGTC ACGGTCCTGA CGCCGTCGTG ATCGCTGGCG  
27301 CCGAGGTACA GGTGCTCGCC CTCGGCGCGA CGTTCGCGGC GCGTGGGATA CGCACGAAGA  
27361 GGCTCGCCGT CTCCCATGCG TTCCATCGC GCCTCATGGA TCCGATGCTG GAAGACTTCC  
27421 AGCGGGTCGC TGCGACGATC GCGTACCGCG CGCCAGACCG CCGGTGGTG TCGAATGTCA  
50 27481 CCGGCCACGT CGCAGGCCCC GAGATCGCCA CGCCCGAGTA TTGGGTCCGG CATGTGCGAA  
27541 GCGCCGTGCG CTTGCGCGAT GGGGCAAAGG CGTTGCATGC CGCGGGTGCC GCCACGTTCC  
27601 TCGAGATTGG CCCGAAGCCG GTCCTGCTCG GGCTATTGCC AGCGTGCTC GGGGAAGCGG  
27661 ACGCGGTCTC CTAGCGCGCG ACCGCTCGGA ATGCGAGGTG GTCCTCGCGG  
27721 CGCTCGGGAC TTGGTATGCC TGGGGGGTG CGCTCGACTG GAAGGGCGTG TTCCCCGATG  
27781 GCGCGCGCGG CGTGGCTCTG CCCATGTATC CATGGCAGCG TGAGCGCCAT TGGATGGACC  
55 27841 TCACCCCGCG AAGCGCCGCG CCTGCAGGA TCGCAGGTCG CTGGCCGCTG CTGGTGTCTG  
27901 GGCTCTGCAT GCGCGCGCT GTGTTGCACC ACGTGCTCTC GATCGGACCA CGCCATCAGC  
27961 CCTTCTCGG TGATCACCTC GTGTTTGGCA AGGTGGTGGT GCGCGGCGCC TTTCATGTCTG  
28021 CCGTGATCCT CAGCATCGCC GCGGAGCGCT GCGCCGAGCG GCGATCGAG CTGACAGGCG  
28081 TGGAGTTCCT GAAGGCGATC GCGATGGAGC CCGACCAGGA GGTGAGCTC CACGCCGTGC



28141 TCACCCCCGA AGCCGCCGGG GATGGCTACC TGTTGAGCT GGCAGCCCTG GCGGCGCCGG  
28201 AGACCGAACG CCGATGGACG ACCCACGCCC GCGGTGCGGT GCAGCCGACA GACGGCGCGC  
28261 CCGGCGCGTT GCCGCGCCTC GAGGTGCTGG AGGACCGCGC GATCCAGCCC CTCGACTTCG  
5 28321 CCGGATTCTT CGACAGGTTA TCGGCGGTGC GGATCGGCTG GGGTCCGCTT TGGCGATGGC  
28381 TGCAGGACGG GCGCGTCGGC GACGAGGCC TCGTTGCCAC CCTCGTGCCG ACCTATCCGA  
28441 ACGCCACGA CGTGGCGCCC TTGCACCGA TCCTGCTGGA CAACGGCTTT GCGGTGAGCC  
28501 TGCTGGCAAC CCGGAGCGAG CCGGAGGACG ACGGGACGCC CCCGCTGCCG TTCGCGTGG  
28561 AACGGGTGCG GTGGTGGCGG GCGCCGGTTG GAAGGGTGCG GTGTGGCGGC GTGCCGCGGT  
10 28621 CGCAGGCATT CCGTGTCTCG AGCTTCGTGC TGGTCGACGA AACTGGCGAG GTGGTCGCTG  
28681 AGGTGGAGGG ATTTGTTTGC CGCCGGGCGC CGCGAGAGGT GTTCTGCGG CAGGAGTCGG  
28741 GCGCGTCGAC TGCAGCCTTG TACCGCCTCG ACTGGCCCGA AGCCCCCTTG CCCGATGCGC  
28801 CTGCGGAACG GATGGAGGAG AGCTGGGTGC TGGTGGCAGC ACCTGGCTCG GAGATGGCCG  
28861 CCGCGCTCGC AACACGGCTC AACCGCTGCG TACTCGCCGA ACCCAAAGGC CTCGAGGCGG  
15 28921 CCCTCGCGGG GGTGTCTCCC GCAGGTGTGA TCTGCCTCTG GGAACCTGGA GCCACGAGG  
28981 AAGCTCCGGC GCGGCGCGAG CGTGTGGCGA CCGAGGGCCT TTCGGTGGTG CAGGCGCTCA  
29041 GGGATCGCGC GGTGCGCCTG TGGTGGGTGA CCACGGGCGC CGTGGCTGTC GAGGCCGGTG  
29101 AGCGGGTGCA GGTGCGCCACA GCGCCGGTAT GGGGCCTGGG CCGGACAGTG ATGCAGGAGC  
29161 GCGCGGAGCT CAGCTGCACT CTGGTGATT TGGAGCCGGA GGTGATGCC GCGCGTTTCA  
20 29221 CTGACGTTCT GCTGCGGGAG CTCGGTCGCG CTGACGACGA GACCCAGTG GTTTCCGTT  
29281 CCGGAGAGCG CCGCGTAGCG CCGCTGGTCA AAGCGACAAC CCCCAGAGG CTCTTGGTCC  
29341 CTGACGACGA ATCCTATCGA CTGGAGCTG GGCAGAAGGG CACATTGGAC CAGCTCCGCC  
29401 TCGCGCGCGC ACAGCGCCGG GCACCCGGCC CCGGCGAGGT CGAGATCAAG GTAACCGCTT  
29461 CCGGGCTCAA CTTCCGGACC GTCCTCGCTG TGCTGGGAAT GTATCCGGGC GACGCTGGGC  
25 29521 CGATGGGCGG AGATTGTGCC AGGTATCGTA CCGCGGTGGG CCAGGGGGTG CACCACCTCT  
29581 CCGTGGCGCA TGCTGTCTAG GCTGTGACG ACCTGGGGAA TCTGCGGCGC GCGAGCGGG  
29641 GGCTGGTGGT CCGGCAGCCT GCAGGGCTGA CTCCGCGCA GGCAGCTACG GTGCCGTTG  
29701 CGTTCCTGAC GGCCTGGCTC GCTGTGACG ACCTGGGGAA TCTGCGGCGC GCGAGCGGG  
29761 TGCTGATCCA TGCTGCGGCC GCGGCGGTGG GCATGGCCGC GGTGCAAATC GCCGATGGA  
30 29821 TAGGGGCCGA GGTGTTCCGC ACGGCGAGCC CGTCCAAGTG GGCAGCGGTT CAGGCCATGG  
29881 CCGTGCCGCG CACGCACATC GCCAGCTCGC GGACGCTGGA GTTTGCTGAG ACGTTCGGC  
29941 AGGTACCCGG CCGCCGGGGC GTGGACGTGG TGCTCAACGC GCTGGCCGGC GAGTTCGTGG  
30001 ACGCGAGCCT GTCCCTGCTG ACGACGGGCG GCGGTTTCTT CGAGATGGGC AAGACCGACA  
30061 TACGGGATCG AGCCGCGGTC GCGGCGGCGC ATCCCGGTGT TCGCTATCGG GTATTGACA  
35 30121 TCCTGGAGCT CGCTCCGGAT CGAACTCGAG AGATCCTCGA GCGCGTGGTC GAGGGCTTTG  
30181 CTGCGGGACA TCTGCGCGCA TTGCCGTGTC ATGCGTTCGC GATCACCAGG GCCGAGGACG  
30241 CGTTTCGGTT CATGGCGCAA CCGCGGATC AGGGCAAGGT CGTGCTGTG CCGGCGCCCT  
30301 CCGCAGCGCC CTTGGCGCCG ACGGGCACCG TACTGCTGAC CCGTGGGCTG GAGCGGTTGG  
30361 GGCTCCACGT GGCCGCTGG CTCGCCCAGC AGGGCGCGCC GCACATGGTG CTCACAGGTC  
40 30421 GCGGGGGCCT GGATACGCCG GCGGCTGCCA AAGCCGTCGC GGAGATCGAA GCGCTCGGCG  
30481 CTCGGGTGAC GATCGCGGCG TCGGATGTG CCGATCGGAA CCGCTGGAG GCTGTGCTCC  
30541 AGGCCATTCC GCGGAGTGG CGCTTACAG GCGTGATCCA TGCAGCCGGA CCGTACGATG  
30601 ATGGTGTGCT TGATGAGCAG ACCACCGACC GCTTCTCGCG GGTGCTGGCA CCGAGGCTGA  
30661 CTGGCGCCTG GAATCTGCAT GAGCTCACGG CCGGCAACGA TCTCGCTTTC TTCGTGCTGT  
45 30721 TCTCCTCCAT GTCGGGGCTC TTGGGCTCGG CCGGGCAGTC CAACTATGCG GCGGCCAACA  
30781 CCTTCCTCGA CGCGCTGGCC GCGCATCGGC GGGCCGAAGG CCTGGCGGCG CAGAGCCTCG  
30841 CGTGGGGCCC ATGGTCCGAC GGAGGCGATG CAGCGGGGCT CAGCGCGGCG CTGCAGGCGC  
30901 GGCTCGTCCG GCATGGGATG GGAGCGCTGT CGCCGCTCA GGGCACCGCG CTGCTCGGGC  
30961 AGGCGCTGGC TCGGCCGGA ACGCAGCTCG GGGCGATGTC GCTCGACGTG CGTGGCGCAA  
50 31021 GCCAAGCTTC GGGAGCGGCA GTGCCGCTG TGTGGCGCGC GCTGGTGCGC GCGGAGGCGC  
31081 GCCATGCGCG GGCTGGGGCG CAGGGGGCAT TGGCCGCGC CCTTGGGGCG CTGCCGAGG  
31141 CGCGTCGCGC CGACGAGGTG CGCAAGGTG TGCAGGCCGA GATCGCGCGC GTGCTTTCAT  
31201 GGGGCGCCG GAGCGCCGTG CCCGTGCAT GCGCGCTGTC GGACTTGGG CTCGACTCGC  
31261 TCACGGCGGT GGAGCTGCGC AACGTGCTCG CCGAGCGGCT GGGTGGCAGC GTGCCGGCGA  
31321 CGCTGGCATT CGATACCCG ACGGTGACG CGTCAACG CTGGCTGCTC GATAAGGTCC  
55 31381 TGGCCGTGGC CGAGCCGAGC GTATCGCCG CAAAGTCGTC GCGCAGGTC GCCCTCGACG  
31441 AGCCATTGCG GGTGATCGGC ATCGGCTGCC GTTCCAGG CCGCGTGACC GATCCGGAGT  
31501 CGTTTTGGCG GCTGCTCGAA GAGGGCAGCG ATGCCGTGCT CGAGGTGCCG CATGAGCGAT  
31561 GGGACATCGA CGCGTTCTAT GATCCGGATC CGGATGTGCG CCGCAAGATG ACGACACGCT  
31621 TTGGCGGCTT CCTGTCCGAT ATCGACCGGT TCGAGCCGGC CTTCTTCGGC ATCTCGCCGC

31681 GCGAAGCGAC GACCATGGAT CCGCAGCAGC GGCTGCTCCT GGAGACGAGC TGGGAGGCGT  
31741 TCGAGCGCGC CGGGATTTTG CCCGAGCGGC TGATGGGCAG CGATACCGGC GTGTTCTGTG  
31801 GGCTCTTCTA CCAGGAGTAC GCTGCGCTCG CCGGCGGCAT CGAGGCGTTC GATGGCTATC  
5 31861 TAGGCACCGG CACCACGGCC AGCGTCGCCT CGGGCAGGAT CTCTTATGTG CTCGGGCTAA  
31921 AGGGGCCGAG CCTGACGGTG GACACCGCGT GCTCCTCGTC GCTGGTTCGCG GTGCACCTGG  
31981 CCTGCCAGGC GCTGCGGCGG GCGGAGTGTT CGGTGGCGCT GGCCGGCGGC GTGGCGCTGA  
32041 TGCTCACGCC GCGGACGTTT GTGGAGTTCA GCCGGCTGCG AGGCCTGGCT CCCGACGGAC  
32101 GGTGCAAGAG CTCTCTCGCC GCAGCCGACG GCGTGGGGTG GAGCGAAGGC TGCGCCATGC  
10 32161 TCCTGCTCAA ACCGCTTCGC GATGCTCAGC GCGATGGGGA TCCGATCCTG GCGGTGATCC  
32221 GCGGCACCGC GGTGAACCAG GATGGGCGCA GCAACGGGCT GACGGCGCCC AACGGGTCTG  
32281 CGCAGCAAGA GGTGATCCGT CCGGCCCTGG AGCAGGCGGG GCTGGCTCCG GCGGACGTCA  
32341 GCTACGTCGA GTGCCACGGC ACCGCCACGA CGTTGGGCGA CCCCATCGAA GTGCAGGCCC  
32401 TGGGCGCCGT GCTGGCACAG GGGCGACCCT CGGACCGGCC GCTCGTGATC GGGTCGGTGA  
15 32461 AGTCCAATAT CGACATACG CAGGCTGCGG CCGGCGTGGC CCGTGTGATC AAGTGTGCGC  
32521 TGGCGCTCGA GCGCGGGCTT ATCCCGAGGA GCCTGCATTT CGACGCGCCC AATCCGCACA  
32581 TTCCGTGGTC GGAGCTCGCC GTGCAGGTGG CCGCCAAACC CGTCGAATGG ACGAGAAACG  
32641 GCGCGCCCGG ACGAGCCGGG GTGAGCTCGT TTGGCGTCAG CCGGACCAAC GCGCAGTGG  
32701 TGCTGGAGGA GGCGCCAGCG GCGGCGTTTG CGCCCGCGGC GGCGCGTTCA GCGGAGCTTT  
20 32761 TCGTGTGTG GCGGAAGAGC GCCGCGGCGC TGGACGCGCA GGCGGCGCGG CTTTCGGCGC  
32821 ATGTCTGTG GCACCCGGAG CTCGGCCTCG GCGACCTGGC GTTCAGCCTG GCGCAGCCC  
32881 GCAGCCCGAT GACGTACCGG CTCGCGGTGG CCGCGACCTC GCGCGAGGCG CTGTCTGCGG  
32941 CGCTCGACAC AGCGGCGCAG GGGCAGGCGC CGCCCGCAGC GGCTCGCGGC CACGCTTCCA  
33001 CAGGCAGCGC CCAAAGGTG GTTTTCGTCT TTTCTGGCCA GGGCTCCCAG TGGCTGGGCA  
25 33061 TGGGCCAAAA GCTCCTCTCG GAGGAGCCG TCTTCCGCGA CGCGCTCTCG GCGTGTGACC  
33121 GAGCGATTCA GGCCGAAGCC GGCTGGTCGC TGCTCGCCGA GCTCGCGGCC GATGAGACCA  
33181 CCTCGCAGCT CGGCCGCATC GACGTGGTGC AGCCGGCGCT GTTCGCGATC GAGGTGCGCG  
33241 TGTCGGCGCT GTGGCGGTGCG TGGGGCGTCG AGCCGATGC AGTGGTAGGC CACAGCATGG  
33301 GCGAAGTGGC GGCCGCGCAC GTCGCCGGCG CCCTGTCTCGT CGAGGATGCT GTAGCGATCA  
30 33361 TCTGCCGGCG CAGCCTGCTG CTGCGGCGGA TCAGCGGCCA AGGCGAGATG GCGGTCTGTCG  
33421 AGCTCTCCCT GGCCGAGGCC GAGGCAGCGC TCCTGGGCTA CGAAGATCGG CTCAGCGTGG  
33481 CCGGTAGCAA CAGCCCGCGA TCGACGGTGC TGGCGGGCGA GCCGCGACGG CTCGAGAGG  
33541 TGCTGGCGAT CCTTGCGGCA AAGGGGGTGT TCTGCCGTCG AGTCAAGGTG GACGTGCCCA  
33601 GCCACAGCCC ACAGATCGAC CCGCTGCGCG ACAGCTATT GGCAGATTG GCGGAGCTCG  
35 33661 AGCCGCGACA AGCGACCGTG TCGATGCGCT CGACGGTGAC GAGCACGATC GTGGCGGGCC  
33721 CGGAGCTCGT GGCGAGCTAC TGGCGGACA ACCTTCGACA GCCGGTGC GC TCCGCCAAG  
33781 CCGTGCAATC GTTGATGGAA GGCGGTGATG GGCTGTTCTG GGAGATGAG CCGCATCCGA  
33841 TCCTGACGAC GTCGGTCGAG GAGATCCGAC GGGCGACGAA GCGGGAGGGA GTCGCGTGG  
33901 GCTCGTTGCG GCGTGGACAG GACGAGCGCC TGTCATGTT GGAGGCGCTG GGAGCGTCT  
40 33961 GGGTACACGG CCAGGCGGTG GGCTGGGAGC GGCTGTTCTC CGCGGGCGGC GCGGGCTCC  
34021 GTCGCGTGCC GTGCCGACC TATCCCTGGC AGCGCGAGCG GTACTGGGTC GAAGCGCGCA  
34081 CCGGCGGCGC GCGGAGCGGC AGCCGCTTTG CTCATGCGGG CAGTCACCCG CTCCTGGGTG  
34141 AAATGCAGAC CCTGTGACAC CAGAGGAGCA CGCGCGTGTG GGAGACGAGC CTGGATCTCA  
34201 AACGGCTGCC GTGGCTCGGC GATCACCAGG TGCAGGGGGC GGTCGTGTT CCGGCGCGCG  
45 34261 CGTACCTGGA GATGGCGCTT TCGTCTGGGG CCGAGGCCTT GGGTGACGGT CCGCTCCAGG  
34321 TCAGCGATGT GGTGCTCGCC GAGGCGCTGG CCTTCGCGGA TGATACGCCG GTGGCGGTGC  
34381 AGGTCATGGC GACCGAGGAG CGACCAGGCC GCCTGCAATT CCACGTTGCG AGCCGGGTGC  
34441 CCGGCCACGG CCGTGCTGCC TTTTGAAGCC ATGCCGCGG GGTGCTGCGC CAGACCGAGC  
34501 GCGCCGAGGT CCCGGCGAGG CTGGATCTGG CCGCGCTTCG TGCCCGGCTT CAGGCCAGCG  
50 34561 CACCCGCTGC GGCTACCTAT GCGGCGCTGG CCGAGATGGG GCTCGAGTAC GGCCAGCGT  
34621 TCCAGGGGCT TGTCGAGCTG TGGCGGGGG AGGGCGAGGC GCTGGGACGT GTGCGGCTCC  
34681 CCGAGGCCGC CGGCTCCCCA GCCGCGTGCC GGCTCCACCC CGCGCTCTTG GATGCGTGCT  
34741 TCCACGTGAG CAGCGCCTTC GCTGACCGC GCGAGGCGAC GCCATGGGTA CCGCTCGAAA  
34801 TCGGCTCGCT GCGGTGGTTC CAGCGGCCGT CCGGGGAGCT GTGGTGTCAT GCGCGGAGCG  
55 34861 TGAGCCACGG AAAGCCAACA CCCGATCGGC GGAGTACCGA CTTTGGGTG GTCCAGACGA  
34921 CCGGCGCGAT CGTCGCCGAG ATCTCCGGG TCGTGGCGCA GCGGCTCGCG GAGGTGTAC  
34981 GCGGCGCGCA AGAAGACGAC TGTTTCATGG AGCCGGCTTG GGAACCGACC GCGGTCCCCG  
35041 GATCCGAGGT CACGGCGGGC CCGTGGCTGC TCATCGGCTC GGGCGGCGG CTCGGCGCTG  
35101 CGCTCTACTC GCGGCTGACG GAAGCTGGCC ATTCGTCGT CCACGCGACA GGGCACGGCA  
35161 CGAGCGCCGC CCGGTTGCAG GCACTCCTGA CCGCGTCTT CGACGGCCAG GCCCCGACGT

35221 CGGTGGTGCA CCTCGGCAGC CTCGATGAGC GTGGCGTGCT CGACGCGGAT GCCCCCTTCG  
35281 ACGCCGATGC CCTCGAGGAG TCGCTGGTGC GCGGCTGCGA CAGCGTGCTC TGGACCGTGC  
35341 AGGCCGTGGC CGGGGCGGGC TTCCGAGATC CTCCGCGGTT GTGGCTCGTG ACACGCGGCG  
5 35401 CTCAGGCCAT CGGCGCCGGC GACGTCTCCG TGGCGCAAGC GCCGCTCCTG GGGCTGGGCC  
35461 GCGTTATCGC CTTGGAGCAC GCCGAGTGC GCTGCGCTCG GATCGACCTC GATCCAGCGC  
35521 GCGCGGACGG AGAGGTCGAT GAGCTGCTTG CCGAGCTGTT GGCCGACGAC GCCGAGGAGG  
35581 AAGTCGCGTT TCGCGGCGGT GAGCGGCGCG TGGCCCGGCT CGTCCGAAGG CTGCCCGAGA  
35641 CCGACTGCCG AGAGAAAATC GAGCCCGCGG AAGGCCGGCC GTTCCGGCTG GAGATCGATG  
35701 GGTCCGGCGT GCTCGACGAC CTGGTGCTCC GAGCCACGGA GCGGCGCCCT CCTGGCCCGG  
10 35761 GCGAGGTCGA GATCGCCGTC GAGGCGGCGG GGCTCAACTT TCTCGACGTG ATGAGGGCCA  
35821 TGGGGATCTA CCTGGGCC CCCTGGGCTC CGGTTGCGCT GGGCGCCGAG TGCTCCGGCC  
35881 GAATTGTGCG GATGGGCGAA GGTGTCTAGA AGCTTCGTAT CGGCCAGGAC GTCGTGGCCG  
35941 TCGCGCCCTT CAGTTTCGGC ACCCACGTCA CCATCGACGC CCGGATGGTC GCACCTCGCC  
36001 CCGCGGCGCT GACGGCCGCG CAGGCAGCCG CGTGCCCGT CGCATTCATG ACGGCCTGGT  
15 36061 ACGGTCTCGT CCATCTGGGG AGGCTCCGGG CCGGCGAGCG CGTGCTCATC CACTCGGCGA  
36121 CGGGGGGCGC CGGGCTCGCT GCTGTGCAGA TCGCCCGCCA CCTCGGCGCG GAGATATTTG  
36181 CGACCGCTGG TACGCGGAG AAGCGGGCGT GGCTGCGCGA GCAGGGGATG GCGCACGTGA  
36241 TGGACTCGCG GTCGCTGGAC GTAGAGATCT AAGTGCTGGC CGCGCAGGAG GCGCAGGGGG  
20 36301 TCGACGTCGT GTTGAACCTG CTGTCTGGCG CCGCGATCGA CGCGAGCCTT GCGACCTCG  
36361 TGCCGGACGG CCGCTTCATC GAGCTCGGCA AGACGGACAT CTATGCAGAT CGCTCGCTGG  
36421 GGCTCGCTCA CTTTAGGAAG AGCCTGTCTT ACAGCGCCGT CGATCTTGCG GGTTTGGCCG  
36481 TGCGTCGGCC CGAGCGCGTC GCAGCGCTGC TGGCGGAGGT GGTGGACCTG CTCGCACGGG  
36541 GAGCGCTGCA GCGCTTCCG GTAGAGATCT TCCCCCTCTC GCGGGCCGCG GACGCGTTCC  
25 36601 GGAAAATGGC GCAAGCGCAG CATCTCGGGA AGCTCGTGCT CGCGCTGGAG GACCCGGACG  
36661 TGCGGATCCG CGTTCCGGGC GAATCCGGCG TCGCCATCCG CGCGGACGGC ACCTACCTCG  
36721 TGACCGGCGG TCTGGGTGGG CTCGGTCTGA GCGTGGCTGG ATGGCTGGCC GAGCAGGGGG  
36781 CTGGGCATCT GGTGCTGGTG GGCCGCTCCG GTGCGGTGAG CGCGGAGCAG CAGACGGCTG  
36841 TGGCCGCGCT CGAGGCGCAC GGCGCGCGTG TCACGGTAGC GAGGGCAGAC GTCGCCGATC  
36901 GGGCGCGAT CGAGCGGATC CTCCGCGAGG TTACCGCGTC GGGGATGCCG CTCGCGGGCG  
30 36961 TCGTTCATGC GGCCGGTATC CTGGACGACG GGCTGCTGAT GCAGCAAACC CCGCGCGGCT  
37021 TCCGCGCGGT CATGGCGCCC AAGGTCCGAG GGGCCTTGCA CCTGCATGCG TTGACACGCG  
37081 AAGCGCCGCT CTCCTTCTTC GTGCTGTACG CTTCCGGAGC AGGGCTCTTG GGCTCGCCGG  
37141 GCCAGGGCAA CTACGCCGCG GCCAACACGT TCCTCGACGC TCTGGCACAC CACCGGAGGG  
37201 CGACGGGCT GCCAGATTG AGCATCGACT GGGGCTGTT CGCGGACGTG GGTTTGGCCG  
35 37261 CCGGGCAGCA AAATCGCGGC GCACGGCTGG TCACCCGCGG GACGCGGAGC CTCACCCCGC  
37321 ACGAAGGGCT GTGGGCGCTC GAGCGTCTGC TCGACGGCGA TCGACCCAG GCCGGGGTCA  
37381 TGCCGTTTCA CGTGCGGCG TGGGTGGAGT TCTACCCGGC GCGGGCATCT TCGCGGAGGT  
37441 TGTCGCGGCT GGTGACGGCA CGGCGCGTGG CTTCCGGTCG GCTCGCCGGG GATCGGGACC  
37501 TGCTCGAACG GCTCGCCACC GCGGAGGCGG GCGCGCGGGC AGGAATGCTG CAGGAGGTG  
40 37561 TGCGCGCGCA GGTCTCGCAG GCTCTCGGCC TCCCCGAAGG CAAGCTCGAC GTGGATGCGC  
37621 CGCTACGAG CCTGGGAATG GACTCGCTGA TGGGGCTAGA GCTGCGCAAC CGCATCGAGG  
37681 CCGTGCTCGG CATCACCATG CCGGCGACCC TGCTGTGGAC CTACCCACG GTGGCAGCGC  
37741 TGAGTGCGCA TCTGGCTTCT CATGTCTCT CTACGGGGGA TGGGGAATCC GCGCGCCCGC  
37801 CGGATACAGG GAACGTGGCT CCAATGACCC ACGAAGTCGC TTCGCTCGAC GAAGACGGGT  
45 37861 TGTTCCGCTT GATTGATGAG TCACTCGCGC GTGCGGGAAA GAGGTGATTG CGTGACAGAC  
37921 CGAGAAGGCC AGCTCCTGGA GCGCTTGGT GAGGTTACTC TGGCCCTTCG CAAGACGCTG  
37981 AACGAGCGCG ATACCCTGGA GCTCGAGAAG ACCGAGCCGA TCGCCATCGT GGGGATCGGC  
38041 TGCCGCTTCC CCGGCGGAGC GGGCACTCCG GAGGCGTTCT GGGAGCTGCT CGACGACGGG  
50 38101 CGCGACGCGA TCCGCGCGCT CGAGGAGCGC TGGGCGCTCG TAGGTGTGCA CCCAGGCGAC  
38161 GACGTACCGC GCTGGGCGGG GCTGCTCACC GAAGCCATCG ACGGCTTCGA CGCCGCGTTC  
38221 TTCGGTATCG CCCCCGGGA GGCACGGTCG CTCGACCCGC AGCATCGCTT GCTGCTGGAG  
38281 GTCGCCTGGG AGGGGTTCTA AGACGCGGCT ATCCCGCCTA GGTCCCTCGT CCGGAGCCCG  
38341 ACCGGCGTGT TCGTCGGCGT CTGCGCCACG GAGTATCTCC ACGCCGCGT CGCGACCCAG  
38401 CCGCGCGAAG AGCGGGACGC GTACAGCACC ACCGGCAACA TGCTCAGCAT CGCCGCGGGA  
55 38461 CCGCTATCGT ACACGCTGGG GCTGCAGGGA CTTGCCTGA CCGTCGACAC GCGTGCTCG  
38521 TCATCGCTGG TGGCCATTCA CCTCGCCTGC CGCAGCCTGC GCGCTCGAGA GAGCGATCTC  
38581 GCGCTGGCGG GAGGGGTCAA CATGCTTCTC TCCCCGACA CGATGCGAGC TCTGGCGCGC  
38641 ACCGAGCGC TGTGCCCCAA TGGCCGTTGC CAGACCTTCG ACGGTCGGC CAACGGGTTT  
38701 GTCCGTGGGG AGGGCTGCGG TCTGATCGTG CTCAAGCGAT TGAGCGACGC GCGGCGGGAT

38761 GGGGACCGGA TCTGGGCGCT GATCCGAGGA TCGGCCATCA ATCAGGACGG CCGGTCGACG  
38821 GGGTTGACGG CGCCCAACGT GCTCGCCCAG GGGGCGCTCT TGCGCGAGGC GCTGCGGAAC  
38881 GCCGGCGTCG AGGCCGAGGC CATCGGTTAC ATCGAGACCC ACGGGGCGGC GACCTCGCTG  
38941 GGCGACCCCA TCGAGATCGA AGCGCTGCGC ACCGTGGTGG GGCCGGCGCG AGCCGACGGA  
5 39001 GCGCGCTGCG TGCTGGGCGC GGTGAAGACC AACCTCGGCC ACCTGGAGGG CGCTGCCGGC  
39061 GTGGCGGGCC TGATCAAGGC TACACTTTCG CTACATCACG AGCGCATCCC GAGGAACCTC  
39121 AACTTTCGTA CGCTCAATCC GCGGATCCGG ATCGAGGGGA CCGCGCTCGC GTTGGCGACC  
39181 GAACCGGTGC CCTGGCCGCG GACGGGCGCG ACGCGCTTCG CGGGAGTGAG CTCGTTCCGG  
10 39241 ATGAGCGGGA CCAACGCGCA TGTGGTGTG GAGGAGGCGC CGGCGGTGGA GCCTGAGGCC  
39301 GCGGCCCCCG AGCGCGCTGC GGAGCTGTTT GTCTGTCTCG CGAAGAGCGT GGCGGCGCTG  
39361 GATGCGCAGG CAGCCCGGCT GCGGGACCAC CTGGAGAAGC ATGTCTGAGT TGGCCTCGGC  
39421 GATGTGGCGT TCAGCCTGGC GACGACGCGC AGCGCGATGG AGCACCAGGCT GCGGCTGGCC  
39481 GCGAGCTCGC GCGAGGCGCT GCGAGGGCGC CTTTCGGCCG CAGCGCAGGG GCATACGCCG  
15 39541 CCGGGAGCCG TGCGTGGGCG GGCTCCGGC GGCAGCGCGC CGAAGGTGGT CTTCTGTGTT  
39601 CCGGCGCAGG GCTCGCAGT GGTGGGCGT GGCCGAAAGC TCATGGCCGA AGAGCCGGTC  
39661 TTCCGGGCGG CGCTGGAGGG TTGCGACCGG GCCATCGAGG CGGAAGCGGG CTGGTCGCTG  
39721 CTCGGGGAGC TCTCCGCCGA CGAGGCCGCC TCGCAGCTCG GGCGCATCGA CGTGGTTACG  
39781 CCGGTGCTCT TCGCCATGGA AGTAGCGCTT TCTGCGCTGT GGCGGTCTGT GGGAGTGGAG  
20 39841 CCGGAAGCGG TGGTGGGCCA CAGCATGGGC GAGGTGGCGG CCGCGCACGT GGCCGGCGCG  
39901 CTGTGCTCG AGGACGCGGT GGCGATCATC TGCCGGCGCA GCCGGTGTCT GCGGCGGATC  
39961 AGCGGTGAGG GCGAGATGGC GCTGGTCGAG CTGTGCTGG AGGAGCCGA GGCGGCGCTG  
40021 CGTGGCCATG AGGGTCTGGT GAGCGTGGCG GTGAGCAACA GCCCGCGCTC GACCGTGCTC  
40081 GCAGGCGAGC CGGCGGCGCT CTCGGAGGTG CTGGCGGCGC TGACGGCCAA GGGGTGTGTT  
25 40141 TGGCGGCAGG TGAAGGTGGA CGTCGCCAGC CATAGCCCGC AGGTGACCCC GCTGCGCGAA  
40201 GAGCTGATCG CCGCGCTGGG GCGGATCCGG CCGCGAGCGG CTGCGGTGCC GATGCGCTCG  
40261 ACGGTGACGG GCGGGGTGAT CGCGGTCCG GAGCTCGGTG CGAGCTACTG GGCGGACAAT  
40321 CTTGCGCAGC CGGTGCGCTT CGCTGCGGCG GCGCAAGCGC TGCTGGAAGG TGGCCCCACG  
40381 CTGTTTCATCG AGATGAGCCC GCACCCGATC CTGGTGCCGC CCCTGGACGA GATCCAGACG  
30 40441 GCGGTGAGC AAGGGGCGCG TGCGGTGGGC TCGCTGCGGC GAGGGCAGGA CGAGCGCGCG  
40501 ACGCTGCTGG AGGCGCTGGG GACGCTGTGG GCGTCCGGCT ATCCGGTGAG CTGGGCTCGG  
40561 CTGTTCCCGG CCGGCGGCAG AGGTGAGCC TGACGCCCGC CCGCTCGCCG CCGCGAAATC GGAGACAGCT  
40621 TGCTGGATCG AGGTGAGCC GCGGACTTTC GCGGACTTTC CTGCCGACCT ATCCCTGGCA GCACGAGCGG  
35 40681 TGGTTCTACC GGACGACTG GCGCGGACTG GCGCGAGAGG GGTGGGGTGG GGTGGGGTGG GCGAGGCGGT CGCTGCAGCG  
40741 CATGGGAGCT GGCTGCTGTT GGCCGACAGG CTTTCATGCGT CCGGCTGACG CTCCACCGTC  
40801 CTGTGACGCG GCGGACTTTC CTGACCCGTG CTTTCATGCGT CCGGCTGACG CTCCACCGTC  
40861 GCGGAGCAGG TATCCGAAGC TGCCAGTCGC CGAAACGACT GGCAGGGAGT CCTCTACCTG  
40921 TGGGGCCTCG ACGCCGCTCGT CGATGCTGGG GCATCGGCCG ACGAAGTCAG CGAGGCTACC  
40981 CGCCGTGCCA CCGCACCCGT CCTTGGGCTG GTTCGATTCC TGAGCGCTGC GCCCATCCT  
40 41041 CCTCGCTTCT GGGTGGTGAC CCGGCGGCA TGCACGGTGG GCGGCGAGCC AGAGGTCTCT  
41101 CTTTGCCAAG CCGCGTTGTG GGGCCTCGCG CGCGTCTGG CGCTGGAGCA TCCCGCTGCC  
41161 TGGGGTGGCC TCGTGGACCT GGATCCTCAG AAGAGCCCGA CCGAGATCGA GCCCTGGTG  
41221 GCGGAGCTGC TTTCGCCGGA CGCCGAGGAT CAACTGGCGT TCCGACGCGG TCGCCGGCAC  
41281 GCAGCACGCC TTGTAGCCGC CCCGCCGGAG GCGGACGTCG CACCGATATC GCTGTCCGCG  
45 41341 GAGGGAAGCT ACCTGGTGAC GGGTGGGCTG GGTGGCCTTG GTCTGCTCGT GGCTCGGTGG  
41401 CTGGTGGAGC GCGGAGAGCA GCCGCCGGAG GCGCGCGCGC GCATCGCAGC GGTGAGGGG  
41461 CAGGCGTCGG GCGGAGAGCA GCCGCCGGAG GCGCGCGCGC GCATCGCAGC GGTGAGGGG  
41521 CTGGAAGCGC AGGGCGCGCG GGTGACCGTG GCAGCGGTGG ATGTGCGCCA GGCCTGATCC  
41581 ATGACGGCGC TGCTGGCCGC CATCGAGCCC CCGTTGCGCG GGGTGGTGCA CGCCGCCGGC  
50 41641 GTCTTCCCCG TGCGTCCCCT GCGGAGACG GACGAGGCC TGCTGGAGTC GGTGCTCCGT  
41701 CCAAGGTGG CCGGAGTGG GCTGCTGCAC CCGCTGCTGC GCGACCGGCC TCTCGACCTG  
41761 TTCGTGCTGT TCTCGTCGGG CCGCGCGGTG TGGGGTGGCA AAGGCCAAGG CGCATACGCC  
41821 GCGGCCAATG CGTTCCTCGA CCGGCTCGCG CACCATCGCC GCGCGCACTC CCTGCGGCG  
41881 TTGAGCCTCG CCTGGGGCCT ATGGGCCGAG GGAGGCGTGG TTGATGCAAA GGCTCATGCA  
55 41941 CGTCTGAGCG ACATCGGAGT CCTGCCCATG GCCACGGGGC CGGCCTTGTC GGCGCTGGAG  
42001 GCGCTGGTGA ACACAGCGC TGTCCAGCGT TCGGTACAC GGTGACTG GCGCGCTTC  
42061 GCGCCGGTCT ATGCCGCGC AGGGCGGCGC AACTTGCTTT CCGCTCTGGT CGCGGAGGAC  
42121 GAGCGCACTG CGTCTCCCCC GGTGCCGACG CCAAACCGGA TCTGGCGCGC CCTGTCCGTT  
42181 GCGGAGAGCC GCTCAGCCCT CTACGAGCTC GTTCGCGGCA TCGTCCCCG GGTGCTGGG  
42241 TTCTCCGACC CCGGCGCGCT CGACGTCCGC CGAGGCTTCG CCGAGCAGGG GCTCGACTCC

42301 CTGATGGCTC TGGAGATCCG TAACCGCCTT CAGCGCGAGC TGGGCGAACG GCTGTGCGCG  
42361 ACTCTGGCCT TCGACCACCC GACGGTGGAG CGGCTGGTGG CGCATCTCCT CACCGACGTG  
42421 CTGAAGCTGG AGGACCGGAG CGACACCCGG CACATCCGGT CGGTGGCGGC GGATGACGAC  
5 42481 ATCGCCATCG TCGGTGCCGC CTGCCGGTTC CCGGGCGGGG ATGAGGGCCT GGAGACATAC  
42541 TGGCGGCATC TGGCCGAGGG CATGGTGGTC AGCACCAGAG TGCCAGCCGA CCGGTGGCGC  
42601 GCGGCGGACT GGTACGACCC CGATCCGGAG GTTCCGGGCC GGACCTATGT GGCCAAGGGG  
42661 GCCTTCCTCC GCGATGTGCG CAGCTTGGAT GCGGCGTTCT TCTCCATCTC CCTCGTGAG  
42721 GCGATGAGCC TGGACCCGCA ACAGCGGCTG TTGCTGGAGG TGAGCTGGGA GGCGATCGAG  
10 42781 CGCGCTGGCC AGGACCCGAT GGCGCTGCGC GAGAGCGCCA CCGGCGTGTT CGTGGGCATG  
42841 ATCGGGAGCG AGCACGCCGA GCGGGTGCAG GGCTTCGACG ACGACGCGGC GTTGCTGTAC  
42901 GGCACCACCG GCAACCTGCT CAGCGTCGCC GCTGGACGGC TGTCGTTCTT CCTGGGTCTG  
42961 CACGCCCCGA CGATGACGGT GGACACCGCG TGCTCGTCGT CGCTGGTGGC GTTGACCTC  
43021 GCCTGCGATT GCCTGCGATT GGTGGAGTGC GACCAAGCAC TGGCCGGCGG GTCCAGCGTG  
43081 CTTTTGTGCG CGCGGTCAAT CGTCGCGGCA TCGCCCATGC GTTTGCTTTC CCTCGTGAG  
15 43141 CGGTGCAAGA CGTTCTCGGC CGCTGCAGAC GGCTTTGCGC GGGCCGAGGG CTGCGCCGTG  
43201 GTGGTGCTCA AGCGGCTCCG TGACGCGCAG CGCGACCGCG ACCCATCCT GGCGGTGGTC  
43261 CGGAGCACGG CGATCAACCA CGATGGCCCC AGCAGCGGGC TCACGGTGCC CAGCGGTCTT  
43321 GCGGCGCAGG CGTTGCTAGG CCAGGCGCTG GCGCAAGCGG GCGTGGCACC GGCCGAGGTC  
43381 GATTTGCTGG AGTGCCACGG GACGGGGACA GCGCTGGGTG ACCCGATCGA GGTGCGAGGC  
20 43441 CTGGGCGCGG TGTATGGCCG GGGCCGCCCC GCGGAGCGGC CGCTCTGGCT GGGCGCTGTC  
43501 AAGGCCAACC TCGGCCACCT GGAGGCCGCG GCGGGCTTGG CCGGCGTGCT CAAGGTGCTC  
43561 TTGGCGCTGG AGCACGAGCA GATTCCGGCT CAACCGAGC TCGACGAGCT CAACCCGCAC  
43621 ATCCCGTGGG CAGAGCTGCC AGTGGCCGTT GTCCGCGCGG CCGTCCCCTG GCCGCGCGGC  
43681 GCGCGCCCGC GTCGTGCAGG CGTGAGCGCT TTCGGCCTGA GCGGGACCAA CGCGCATGTG  
25 43741 GTGTTGGAGG AGGCGCCGGC GGTGGAGCCT GAGGCCGCGG CCCCAGAGCG CGCTCGGAG  
43801 CTGTTCTGCC TGTGCGCGAA GAGCGTGGCG GCGCTGGATG CGCAGGCAGC CCGGCTGCGG  
43861 GATCATCTGG AGAAGCATGT CGAGCTTGGC CTCGGCGATG TGGCGTTTCA CCTGGCGACG  
43921 ACGCGCAGCG CGATGGAGCA CCGGCTGGCG GTGGCCGCGA GCTCGCGCGA GGCGCTGCGA  
43981 GGGGCGCTTT CGGCCGCGAG GCAGGGCGAT ACGCCGCGCG GAGCCGTGCG TGGGCGGGCC  
30 44041 TCCGGCGGCA TCCGCGCGAA GCGGCTTTC GTGTTTCCC GCGAGGGCTC GCAGTGGGTG  
44101 GGCATGGGCC GAAAGCTCAT GGCCGAAGAG CCGGTCTTCC GGGCGGCGT GAGGGTTGCG  
44161 GACCGGGCCA TCGAGGCGGA AGCGGGCTGG TCGCTGCTCG GGGAGCTCTC CGCCGACGAG  
44221 GCCGCTTCGC AGCTCGGGCG CATCGACGTG GTTCAGCCGG TGCTCTTCGC CGTGGAAGTA  
44281 GCGCTTTTCA CGCTGTGGCG GTCGTGGGGA GTGGAGCCGG AAGCGGTGGT GGGCCACAGC  
35 44341 ATGGGCGAGG TTGCGGCGCG GCACGTGGCC GCGCGCTGT CGCTCGAGGA TGCGGTGGCG  
44401 ATCATCTGCC GGCGCAGCG GCTGCTGGCG GCGATCAGCG GTCAGGGCGA GATGGCGCTG  
44461 GTCGAGCTGT CGCTGGAGGA GGCCGAGGCG GCGCTGCGTG GCCATGAGG TCGGCTGAGC  
44521 GTGGCGGTGA GCAACAGCCC GCGCTCGACC GTGCTCGCAG GCGAGCCGGC GGCGCTCTCG  
44581 GAGGTGCTGG CCGCGCTGAC GGCCAAGGGG GTGTTCTGGC GGCAGGTGAA GGTGGACGTC  
40 44641 GCCAGCCATA GCGCGCAGGT CGACCCGCTG CGCGAAGAGC TGGTCGCGGC GCTGGGAGCG  
44701 ATCCGCGCGC GAGCGGCTGC GGTGCCGATG CGCTCGACGG TGACGGGCGG GGTGATTGCG  
44761 GGTCCGAGC TCGGTGCGAG GCTTCGCGAG GACAATCTT GGCAGCCGCT GCGCTTCGCT  
44821 GCGGCGGCGC AAGCGCTGCT GGAAGGTGGC CCCACGCTGT TCATCGAGAT GAGCCCGCAC  
44881 CCGATCCTGG TGCCGCTCTT GGACGAGATC CAGACGGCGG TCGAGCAAGG GGGCGCTGCG  
45 44941 GTGGGCTCGC TGCGGCGAGG GCAGGACGAG CGCGCGACGC TGCTGGAGGC GCTGGGGACG  
45001 CTGTGGGCGT CCGGCTATCC GGTGAGCTGG GCTCGGCTGT TCCCCGCGGG CCGCAGGCGG  
45061 GTTCCGCTGC CGACCTATCC CTGGCAGCAC GAGCGGTACT GGATCGAGGA CAGCGTGCTG  
45121 GGGTCGAAGC CCTCGCTGCG GCTTCGCGAG CTTTATAACG GCGCCACGGA CCATCCGCTG  
50 45181 CTCGGGGCTC CATTGCTCGT CTCGGCGCGA CCCGGAGCTC ACTTGTGGGA GCAAGCGCTG  
45241 AGCGACGAGA GGCTATCCTA TCTTTCGGA CATAGGGTCC ATGGCGAAGC CGTGTGCCCC  
45301 AGCGCGGCGT ATGTAGAGAT GGCGCTCGCC GCGGCGTAG ATCTCTATGG CGCGGCGACG  
45361 CTGGTGCTGG AGCAGCTGGC GCTCGAGCGA GCGCTCGCGG TGCTTCCGA AGGCGGACGC  
45421 ATCGTGCAAG TGGCCCTCAG CGAAGAAGGG CCCGGTCCGG CCTATTCCA GGTATCGAGC  
45481 CGTGAGGAGG CAGGTAGAAG CTGGGTTCGG CACGCCACGG GGCACGTGTG TAGCGACGAG  
55 45541 AGCTCAGCAG TGGGAGCGTT GAAGGAAGCT CCGTGGGAGA TTCAACAGCG ATGTCCGAGC  
45601 GTCCTGTCGT CGGAGGCGCT CTATCCGCTG CTCAACGAGC ACGCCCTCGA CTATGGCCCC  
45661 TGCTTCCAGG GTGTGGAGCA GGTGTGGCTC GGCACGGGGG AGGTGCTCGG CCGGCTACGC  
45721 TTGCCAGAAG ACATGGCATC CTCAAGTGGC GCCTATCGGA TTCATCCCGC CTGTGTTGGT  
45781 GCATGTTTTT AAGTGCTGAC CCGCTGCTC ACCACGCGG AATCCATCGA GATTCGGAGG

45841 CGGCTGACGG ATCTCCACGA ACCGGATCTC CCGCGGTCCA GGGCTCCGGT GAATCAAGCG  
45901 GTGAGTGACA CCTGGCTGTG GGACGCCGCG CTGGACGGTG GACGGCGCCA GAGCGCGAGC  
45961 GTGCCCCGTCG ACCTGGTGCT CGGCAGCTTC CACGCGAAGT GGGAGGTCAT GGATCGCCTC  
5 46021 GCGCAGACGT ACATCATCCG CACTCTCCGC ACATGGAACG TCTTCTGCGC TGCTGGAGAG  
46081 CGTCACACGA TAGACGAGTT GCTCGTCAGG CTCCAAATCT CTGCTGTCTA CAGGAAGGTC  
46141 ATCAAGCGAT GGATGGATCA CCTTGTGCGG ATCGGCGTCC TTGTAGGGGA CCGAGAGCAT  
46201 CTTGTGAGCT CTCAGCCGCT GCCGAGCAT GATTGGGCGG CCGTGCTCGA GGAGCCGCG  
46261 ACGGTGTTCC CCGACCTCCC AGTCCTACTT GAGTGGTGCA AGTTTGCCGG GGAACGGCTC  
10 46321 GCGGACGTGT TGACCGGGAA GACGCTGGCG CTCGAGATCC TCTTCCCTGG CCGTCCGTTT  
46381 GATATGGCGG AGCGAATCTA TCAAGATTCC CCCATCGCCC GTTACTCGAA CCGCATCGTG  
46441 CGCGGTGTCG TCGAGTCGGC GGCGCGGGTG GTAGCACCGT CCGGAACGTT CAGCATCTTG  
46501 GAGATCGGAG CAGGGACGGG CGCGACCACC GCCGCCGTCC TCCCGGTGTT GCTGCCTGAC  
46561 CCGACAGAAT ACCATTTTAC CGATGTTTCT CCGCTCTTCC TTGCTCGTGC GGAGCAAAGA  
15 46621 TTTTCGAGATC ATCCATTCTT GAAGTATGTT ATTCTGGATA TCGACCAGGA GCCAGCTGGC  
46681 CAGGGATACG CACATCAGAA GTTCGACGTC ATCGTCGCGG CCAACGTCAT CCATGCGACC  
46741 CGCGATATAA GAGCCACGGC GAAGCGTCTC CTGTGCTTGC TCGCGCCCGG AGGCCTTCTG  
46801 GTGCTGGTCG AGGGCACAGG GCATCCGATC TGGTTCGATA TCACCACGGG ATTGATCGAG  
46861 GGGTGGCAGA AGTACGAAGA TGATCTTCGT ACCGACCATC CGCTCCTGCC TGCTCGGACC  
20 46921 TGGTGTGACG TCCTGCGCCG GGTAGGCTTT CCGGATGCCG TGAGTCTGCC AGGCGACGGA  
46981 TCTCCGGCGG GGATCCTCGG ACAGCACGTG ATCCTCTCGC GCGCTCCGGG CATAGCAGGA  
47041 GCCGCTTGTG ACAGCTCCGG TGAGTCGGCG ACCGAATCGC CGGCCGCGCG TGCAGTACGG  
47101 CAGGAATGGG CCGATGGCTC CGCTGACGGC GTCCATCGGA TGGCGTTGGA GAGAATGTAC  
47161 TTCCACCGCC GGCCGGGCGG GCAGGTTTGG GTCCACGGTC GATTGCGTAC CCGTGGAGGC  
25 47221 GCGTTTACGA AGGCGCTCAC TGGAGATCTG CTCCTGTTCC AAGAGACCGG GCAGGTCGTG  
47281 GCAGAGGTTT AGGGGCTCCG CTCGAGGCTT CTGCTTTCGC GCCGCGGGAC  
47341 CCGCGGGAAG AGTGGTTGTA CGCGTTGGAA TGGCAGCGCA AAGACCCAT ACCAGAGGCT  
47401 CCGGCAGCCG CGTCTTCTTC CACCGCGGGG GCTTGGCTCG TGCTGATGGA CCGGCGCGG  
47461 ACAGGCGCTG CGTCTGATC GCTGCTGGAA GGGCGAGGCG AGGCGTGCGT GCGCGTCGTC  
30 47521 GCGGGTACGG CATAAGCCTG CCTGCGCGCG GGGCTGTATC AAGTCGATCC GGCGCAGCCA  
47581 GATGGCTTTC ATACCCGTCT TCGCGAGGAG TTCCGGCGAGG ACCGGATGTG CCGCGCGGTA  
47641 GTGCATATGT GGAGCCTTGA TGCGAAGGCA GCAGGGGAGA GGAGTCGCTT TGGTGCGCG  
47701 CAGGCCGATC AACTCCTGGG GAGCCTGAGC GCGCTTCTC TGGTGACGGC TGCGTGCGG  
35 47761 CCGAGGTGGC GCAACATGCC GCGACTTTGG CTCTTGACCC GCGCCGTGCA TGCGTGCGG  
47821 GCGGAGGACG CAGCGGCCTC GGTGGCGCAG GCGCCGGTGT GGGGCTCCG TCGGACGCTC  
47881 GCGCTCGAGC ATCCAGAGCT GCGGTGCACG CTCGTGGACG TGAACCCGGC GCCGTCTCCA  
47941 GAGGACGCGC CTGCACTCGC GGTGGAGCTG GGGGCGAGCG ACAGAGAGGA CCAGATCGCA  
48001 TTGCGCTCGA ATGGCCGCTA CGTGGCGCGC CTCGTGCGGA GCTCCTTTTC CGGCAAGCCT  
48061 GCTACGGATT GCGGCATCCG GCGGACGGC AGTTATGTGA TCACCGATGG CATGGGGAGA  
40 48121 GTGGGGCTCT CCGTCCGCGA ATGGATGGTG ATGCAGGGG CCCGCCATGT GGTGCTCGTG  
48181 GATCGCGCG GCGCTTCCGA CGCCTCCCGG GATGCCCTCC GGTCCATGGC CGAGGCTGGC  
48241 GCAGAGGTGC AGATCGTGA GCGCGACGTG GCTCGGCGCG TCGATGTCGC TCGGCTTCTC  
48301 TCGAAGATCG AACCCTCGAT GCCCGCGCTT CCGGGGATCG TGTACGTGGA CCGGACCTTC  
48361 CAGGGCGACT CCTCGATGCT GGAGCTGGAT GCCCATCGCT TCAAGGAGTG GATGTATCCC  
45 48421 AAGGTGCTCG GAGCGTGGAA CCTGCACGCG CTGACCAGGG ATAGATCGCT GGACTTCTT  
48481 GTCCTGTACT CCTCGGGCAC CTCGCTTCTG GGCTTGCCCG GACAGGGGAG CCGCGCCGCC  
48541 GGTGACGCCT TCTTGGACGC CATCGCGCAT CACCGGTGTA GGCTGGCCCT CACAGCGATG  
48601 AGCATCAACT GGGGATTGCT CTCGAAGCA TCATCGCCGG CGACCCCGAA CGACGGCGGC  
48661 GCACGGCTCC AATACCGGGG GATGGAAGGT CTCACGCTGG AGCAGGGAGC GGAGGCGCTC  
50 48721 GGGCGCTTGC TCGCACAACC CAGGGCGCAG GTAGGGGTAA TGCGGCTGAA TCTGCGCCAG  
48781 TGGCTGGAGT TCTATCCCAA CGCGGCCCGA CTGGCGCTGT GGGCGGAGTT GCTGAAGGAG  
48841 CGTGACCGCA CCGACCGGAG CGCGTCGAAC GCATCGAACC TGCGCGAGGC GCTGCAGAGC  
48901 GCCAGGCCCG AAGATCGTCA GTTGGTCTG GAGAAGCACT TGAGCGAGCT GTTGGGGCGG  
48961 GGGCTGCGCC TTCCGCCGGA GAGGATCGAG CCGCACGTGC CGTTCAGCAA TCTCGGCATG  
55 49021 GACTCGTTGA TAGGCCTGGA GCTCCGCAAC CGCATCGAG CCGCGCTCGG CATCACCGTG  
49081 CCGGCGACCC TGCTATGGAC TTACCCTACC GTAGCAGTC TGAGCGGGAA CCTGCTAGAT  
49141 ATTCTGTTCC CGAATGCCGG CGCGACTCAC GCTCCGGCCA CCGAGCGGGA GAAGAGCTTC  
49201 GAGAACGATG CCGCAGATCT CGAGGCTCTG CCGGGTATGA CCGACGAGCA GAAGGACGCG  
49261 TTGCTCGCCG AAAAGCTGGC GCAGCTCGCG CAGATCGTTG GTGAGTAAGG GACTGAGGGA  
49321 GTATGGCGAC CACGAATGCC GGAAGCTTG AGCATGCCCT TCTGCTCATG GACAAGCTTG

49381 CGAAAAAGAA CGCGTCTTTG GAGCAAGAGC GGACCGAGCC GATCGCCATC ATAGGTATTG  
49441 GCTGCCGCTT CCCCGGCGGA GCGGACACTC CGGAGGCATT CTGGGAGCTG CTCGACTCGG  
49501 GCCGAGACGC GGTCACGCCG CTCGACCGGC GCTGGGCGCT GGTCGGCGCT CATCCCAGCG  
5 49561 AGGAGGTGCC GCGCTGGGCC GGACTGCTCA CCGAGGCGGT GGACGGCTTC GACGCCGCGT  
49621 TCTTTGGCAC CTCGCTCGG GAGGCGCGGT CGCTCGATCC TCAGCAACGC CTGCTGCTGG  
49681 AGGTCACCTG GGAAGGGCTC GAGGACGCCG GCATCGCACC CCAGTCCCTC GACGGCAGCC  
49741 GCACCGGGGT ATTCTTGGGC GCATGCAGCA GCGACTACTC GCATACCGTT GCGCAACAGC  
49801 GCGCGAGGA GCAGGACGCG TACGACATCA CCGGCAATAC GCTCAGCGTC GCCGCCGGAC  
49861 GGTGTCTTA TACGCTAGGG CTGCAAGGAC CCTGCCTGAC CGTCGACACG GCCTGCTCGT  
10 49921 CGTCGCTCGT GGCCATCCAC CTTGCCTGCC CGAGCCTGCG CGCTCGCAG AGCGATCTCG  
49981 CGCTGGCGGG GGGCGTCAAC ATGCTCCTTT CGTCCAAGAC GATGATAATG CTGGGGCGCA  
50041 TCCAGGCGCT GTCGCCCCGAT GGCCACTGCC GGACATTCTGA CGCCTCGGCC AACGGGTTTCG  
50101 TCCGTGGGGA GGGCTGCGGT ATGGTCGTGC TCAAACGGCT CTCCGACGCC CAGCGACATG  
50161 GCGATCGGAT CTGGGCTCTG ATCCGGGGTT CGGCCATGAA TCAGGATGGC CGGTGACAG  
15 50221 GGTGTGATGG ACCCAATGTG CTCGCTCAGG AGGCGCTCTT ACGCCAGGCG CTGCGAGAGCG  
50281 CTCGCGTCGA CGCCGGGGCC ATCGATTATG TCGAGACCCA CGGAACGGGG ACCTCGCTCG  
50341 GCGACCCGAT CGAGGTCGAT GCGCTGCGTG CCGTGATGGG GCCGGCGCGG GCCGATGGGA  
50401 GCCGCTGCGT GCTGGGCGCA GTGAAGACCA ACCTCGGCCA CCTGGAGGGC GCTGCAGGCG  
50461 TGGCGGGTTT GATCAAGGCG GCGCTGGCTC TGCACCACGA ATCGATCCCG CGAAACCTCC  
20 50521 ATTTTCACAC GCTCAATCCG CGGATCCGGA TCGAGGGGAC CGCGCTCGCG CTGGCGACGG  
50581 AGCCGGTGCC GTGGCCGCGG GCGGCGCCAG CGCGCTTCGC GGGGGTGAGC GCGTTCGGCC  
50641 TCAGCGGCAC CAACGTCCAT GTCGTGCTGG AGGAGGCGCC GGCCACGGTG CTCGCACCGG  
50701 CGACGCCGGG GCGCTCAGCA GAGCTTTTGG TGCTGTGCGC GAAGAGCACC CCGCGCTGG  
50761 ACGCACAGGC GCGCGGGCTC TCAGCGCACA TCGCCGCGTA CCCGGAGCAG GGCCCTGGAG  
25 50821 ACGTCGCGTT CAGCCTGGTA GCGACGCGGA GCCCGATGGA GCACCGGCTC GCGGTGGCGG  
50881 CGACCTCGCG CGAGGCGCTG CGAAGCGCGC TGGAAGCTGC GCGCGAGGGG CAGACCCCGG  
50941 CAGGCGCGGC GCGCGGCGAG GCCGCTTCCT CGCCCGGCAA GCTCGCCTTC CTGTTGCGCG  
51001 GGCAGGGCGC GCAGGTGCCG GGCATGGGCC GTGGGTTGTG GGAGGCGTGG CCGGCGTTCC  
51061 GCGAGACCTT CGACCGGTGC GTCACGCTCT TCGACCGGGA GCTCCATCAG CCGCTCTGCG  
30 51121 AGGTGATGTG GGCCGAGCCG GGCAGCAGCA GGTCGTGCTT GCTGGACCAG ACGGCATTCA  
51181 CCCAGCCGGC GCTCTTTGCG CTGGAGTACG CGCTGGCCGC GCTCTTCCGG TCGTGGGGCG  
51241 TGGAGCCGGA GCTCATCGCT GGCCATAGCC TCGGCGAGCT GGTGGCCGCC TCGTGGCGG  
51301 GTGTGTTCTC CCTCGAGGAC ATGGTATCGA TCGCCGCGCC GGAGGCGCAG GTGGCTGCCG  
35 51361 CGCTGCCGGC CGGCGGTGCG ATGGTATCGA TCGCCGCGCC CAATGGGCGG GAGCAGGTGG  
51421 CCGTGGCGCC GCACGCAGCG TCGGTGTCGA TCGCGGCAGT CAATGGGCGG GAGCAGGTGG  
51481 TGATCGCGGG CGCCGAGAAA TTCGTGCAGC AGATCGCGGC GCGGTTTCGCG GCGCGGGGGG  
51541 CGCGAACCAG ACCGCTGCAT GTTTCGCACG CGTTCCACTC GCCGCTCATG GATCCGATGC  
51601 TGGAGGCGTT CCGGCGGGTG ACCGAGTCGG TGACGTATCG GCGGCCTTCG ATGGCGCTGG  
40 51661 TGAGCAACCT GAGCGGGAAG CCTGCACGG ATGAGGTGTG CGCGCCGGGT TACTGGGTGC  
51721 GTCACGCGCG AGAGGCGGTG CGCTTCGCGG ACGGCGTGAA GGCGCTGCAC CGGCGCGGTG  
51781 CCGGCATCTT CGTCGAGGTG GGCCCGAAGC CGGCGCTGCT CGGCCCTTTG CCGGCGTGGC  
51841 TGCCGGATGC CAGGCCGGTG CTGCTCCAG CGTCGCGCGC CGGGCGTGAC GAGGCTGCGA  
51901 GCGCGCTGGA GCGCTGGGT GGGTTCTGGG TCCTCGGTGG ATCGGTCAAC TGGTCGGGTG  
51961 TCTTCCCTTC GGGCGGACGG CCGGTACCG TGCCAACCTA TCCCTGGCAG CGCGAGCGTT  
45 52021 ACTGGATCGA AGCGCCGGTC GATGGTGAGG CGGACGGCAT CGGCCGTGCT CAGGCGGGGG  
52081 ACCACCCCTT TCTGGGTGAA GCCTTTTCCG TGTCGACCCA TGCCGGTCTG CGCCTGTGGG  
52141 AGACGACGCT GGACCGAAAG CCGCTGCCGT GGCTCGGCGA GCACCGGGCG CAGGGGGAGG  
52201 TCGTGTTCCT TGGCGCCGGG TACCTGGAGA TGGCGCTGTC GTCGGGGGCC GAGATCTTGG  
52261 GCGATGGACC GATCCAGGTC ACGGATGTGG TGCTCATCGA GACGCTGACC TTCGCGGGCG  
50 52321 ATACGGCGGT ACCGGTCCAG GTGGTGACGA CCGAGGAGCG ACCGGGACGG CTGCGGTTCC  
52381 AGGTAGCGAG TCGGGAGCCG GGGGCACGTC GCGGTCCTT CCGGATCCAC GCCCGCGGCG  
52441 TGCTGCGCCG GGTGCGGCGC GCCGAGACCC CGGCGAGGTT GAACCTCGCC CCCCTCGCG  
52501 CCCGCTTCA TGCCGCGGTG CCCGCTGCGG CTATCTATGG GCGGCTCGCC GAGATGGGGC  
52561 TTCAATACGG CCCGGCGTTG CCGGGGCTCG CCGAGCTGTG GCGGGGTGAG GCGGAGGCGC  
55 52621 TGGGCAGAGT GAGACTGCCT GAGTCCGCCG GCTCCGCGAC AGCCTACCAG CTGCATCCGG  
52681 TGCTGCTGGA CCGTGCCTG CAAATGATTG TTGGCGCGTT CGCCGATCGC GATGAGGCGA  
52741 CCGCGTGGGC GCGGCTGGAG GTGGGCTCGG TCGGCTGTT CCAGCGGTCT CCTGGGGAGC  
52801 TATGGTGCCA TGCGCGCGTC GTGAGCGATG GTCAACAGG CCCCAGCCCG TGGAGCGCG  
52861 ACTTTGAGTT GATGGACGGT ACGGGCGCGG TGCTCGCCGA GATCTCCCG CTGGTGGTGG



52921 AGCGGCTTGC GAGCGGTGTA CGCCGGCGCG ACGCAGACGA CTGGTTCCTG GAGCTGGATT  
52981 GGGAGCCCGC GGCGCTCGAG GGGCCCAAGA TCACAGCCGG CCGGTGGCTG CTGCTCGGCG  
53041 AGGGTGGTGG GCTCGGGCGC TCGTTGTGCT CAGCGCTGAA GGCCGCCGGC CATGTCTGCG  
53101 TCCACGCCGC GGGGGACGAC ACGAGCGCTG CAGGAATGCG CGCGCTCCTG GCCAACCGCT  
53161 TCGACGGCCA GGCCCCGACG GCCGTGGTGC ACCTCAGCAG CCTCGACGGG GCGGGCCAGC  
53221 TCGACCCGGG GCTCGGGGCG CAGGGCGCGC TCGACGCGCC CCGGAGCCCA GATGTCGATG  
53281 CCGATGCCCT CGAGTCGGCG CTGATGCGTG GTTGCAGACG CGTGCTCTCC CTGGTGCAAG  
53341 CGCTGGTCGG CATGGACCTC CGAAATGCGC CGCGGCTGTG GCTTTTGACC CGCGGGGCTC  
53401 AGGCGGCCGC CGCCGGCGAT GTCTCCGTGG TGCAAGCGCC GCTGTTGGGG CTGGGCCGCA  
53461 CCATCGCCTT GGAGCACGCC GAGCTGCGTG GTATCAGCGT CGACCTCGAT CCAGCCCAGC  
53521 CTGAAGGGGA AGCCGATGCT TTGCTGGCCG AGCTACTTGC AGATGATGCC TGGACGGAGG  
53581 TCGCGCTGCG CCGTGGCGAG CGGTTTGTG CGCGGCTCGT CCACCGGCTG CCCGAGGCTC  
53641 AACGCCGGGA GAAGATCGCG CCCGCCGGTG ACAGGCCGTT CCGGCTAGAG ATCGATGAAC  
53701 CCGGCGTGCT GGACCAACTG GTGCTCCGGG CCACGGGGCG GCGCGCTCCT GGTCCGGGCG  
53761 AGGTCGAGAT CGCCGTCGAA GCGGCGGGG TCGACTCCAT CGACATCCAG CTGGCGGTGG  
53821 GCGTTGCTCC CAATGACCTG CCTGAGGAG AAATCGAGCC GTGCGTGCTC GGAAGCGAGT  
53881 GCGCCGGGCG CATCGTCGCT GTGGGCGAGG GCGTGAACGG CCTGTGTTGG GCCCAGCCGG  
53941 TGATCGCCCT TCGGGCGGGA GTATTGCTA CCCATGTCAC CACGTCGGCC ACGTGGTGT  
54001 TGCTCGGCC TCTGGGGCTC TCGCGACCG AGGCGGCCGC GATGCCCTC GCGTATTTGA  
54061 CGGCTTGGTA CGCCCTCGAC AAGGTCGCCC ACCTGCAGGC GGGGAGCGCG GTGCTGATCC  
54121 GTGCGGAGGC CGGTGGTATC GGTCTTTCG CGGTGCGATG GGCGCAGCGC GTGGGCGCCG  
54181 AGGTGTATGC GACCGCCGAC ACGCCCGAGA AACGTGCCTA CCTGGAGTCG CTGGGCGTGC  
54241 GGTACGTGAG CGATTCCCGC TCGGGCCGGT TCGCCGAGA CGTGCATGCA TGGACGGACG  
54301 GCGAGGGTGT GGACGTCGTG CTCGACTCGC TTTCCGGCGA GCACATCGAC AAGAGCCTCA  
54361 TGGTCTTGCG CGCCTGTGGC CGCCTTGTGA AGCTGGGCGAG GCGCGACGAC TCGCCGACA  
54421 CGCAGCCTGG GCTGCCGCCG CTCCTACGGA ATTTTTCCTT CTCGAGGTG GACTTGCGGG  
54481 GAATGATGCT CGATCAACCG CGAGGATCC GTGCGCTCCT CGACGAGTG TCCCTCACGC  
54541 TCGCAGCCGG TGCCATCAGC CCACTGGGGT CGGGGTTGCG CGTTGGCGGA AGGATGGCGC  
54601 CACCGCCGGT CGAGACCTT CCGATCTCTC GCGCAGCCGA GGCATTCCGG AGGATGGCGC  
54661 AAGGACAGCA TCTCGGGAAG CTCGTGCTCA CGCTGGACGA CCCGAGGTG CCGATCCGCG  
54721 CTCCGGCCGA ATCCAGCGTC GCGTCCGCG GTGGCCGAT GGCTGGCCGA GCGGGGCGCG GGGCACTGG  
54781 TGGGTGGGCT CGGTCTGCGT GCGGCGAGCG CAGAGCAGCG AGCCGCCGTG GCGGCGCTAG  
54841 TGCTGGTGGG CCGCTCCGGT ACAGGTCGGA AAGCGGATGT CGCCGATCGG TCACAGATCG  
54901 AGGCCACCG CGCGCGCGTC ACGGTGGCGA GCGGCGGCTC GCGGGGTGTC GTGCATGCGG  
54961 AGCGGGTCCT CCGCGAGGTT ACCGCGTCGG GGATGCCGCT GCGGGGTGTC GTGCATGCGG  
55021 CAGGTCTTGT GGATGACGGG CTGCTGATGC AGCAGACTCC GCGCGGGCTC CGCACGGTGA  
55081 TGGGACCTAA GGTCCAGGGA GCCTTGCACT TGCACACGCT GACACGCGAA GCGCCTCTTT  
55141 CCTTCTTCGT GCTGTACGCT TCTGACGCT GGCTGTTCCG CTCGCCAGGC CAGGGCAACT  
55201 ATGCCGCGAG CAACGCGTTC CTCGACGCC TTTCCGATCA CCGCAGGGCG CACGGCCTGC  
55261 CGGCGCTGAG CATCGACTGG GGCATGTTCA CGGAGGTGGG GATGGCCGTT GCGCAAGAAA  
55321 ACCGTGGCGC GCGGCTGATC TCTCGCGGGA TCGGGGGCAT CACCCCGAT GAGGGTCTGT  
55381 CAGCTCTGGC GCGCTTGCTC GAGGGTGATC GCGTGACAG GGGGGTGATA CCGATCACTC  
55441 CGCGGCAGTG GGTGGAGTTC TACCCGGCAA CAGCGGCCTC ACGGAGGTG TCGCGGCTGG  
55501 TGACCACGCA GCGCGCGGTT GCTGATCGGA CCGCCGGGGA TCGGGACCTG CTCGAACAGC  
55561 TTGCTCGGC TGAGCCGAGC GCGCGGGCGG GGCTGCTGCA GGACGTCGT GCGGTGCAGG  
55621 TCTCGCATGT GCTGCGTCTC CCTGAAGACA AGATCGAGGT GGATGCCCCG CTCTCGAGCA  
55681 TGGGCATGGA CTCGCTGATG AGCCTGGAGC TCGCAACCG CATCGAGGCT GCGCTGGGCG  
55741 TCGCCGCGCC TGCAGCCTTG GGGTGGACGT ACCCAACGGT AGCAGCGATA ACGCGCTGGC  
55801 TGCTCGACGA GCGCTCGCC GTCCGGCTTG GCGGCGGGTC GGACACGGAC GAATCGACGG  
55861 CAAGCGCCGG ATCGTTTCGT CACGTCTCCT GCTTTCGTCC TGTCGTCAAG CCGCGGGCTC  
55921 GTCTCTTCTG TTTTCACGGT TCTGGCGGCT CGCCCGAGGG CTTCGGTTCC TGGTCGGAGA  
55981 AGTCTGAGTG GAGCGATCTG GAAATCGTGG CCATGTGGCA CGATCGCAGC CTCGCTCCG  
56041 AGGACGCGCC TGGTAAGAAG TACGTCCAAG AGGCGGCCTC GCTGATTAG CACTATGCAG  
56101 ACGCACCGTT TGCGTTAGTA GGGTTCAGCC TGGGTGTCCG GTTCGTATG GGGACAGCCG  
56161 TGGAGCTCG TAGTCGTTCC GCGCACCCG CTCCGCTGCG CGTTTTTTCG TTGGGCGGCA  
56221 GCTTGATCTC TTCTTCAGAG ATACCCCGG AGATGGAGAC CGATATAATA GCCAAGCTCT  
56281 TCTTCCGAAA TGCCGCGGGT TTCGTCCGAT CCACCAACA AGTTAGGCC GATGCTCGCG  
56341 CAGACAAGGT CATCACAGC ACCATGGTGG CTCCGGCCCC CGGGACTCG AAGGAGCCG  
56401 CCTCGAAGAT CCGGTCCCT ATCGTCGCA TCGCCGGCTC GGACGATGTG ATCGTGCCCT



56461 CAAGCGACGT TCAGGATCTA CAATCTCGCA CCACGGAGCG CTTCTATATG CATCTCCTTC  
56521 CCGGAGATCA CGAGTTTCTC GTCGATCGAG GCGCGGAGAT CATGCACATC GTCGACTCGC  
56581 ATCTCAATCC GCTGCTCGCC GCGAGGACGA CGTCGTCAGG CCGCGCGTTC GAGGCAAAAT  
56641 GATGGCAGCC TCCCTCGGGC GCGCGAGATG GTTGGGAGCA GCGTGGGTGC TGGTGGCCGG  
5 56701 CGGCAGGCAG CGGAGGCTCA TGAGCCTTCC TGGAAGTTTG CAGCATAGGA GATTTTATGA  
56761 CACAGGAGCA AGCGAATCAG AGTGAGACGA AGCCTGCTTT CGACTTCAAG CCGTTCGCGC  
56821 CTGGGTACGC GGAGGACCCG TTTCCCGCGA TCGAGCGCCT GAGAGAGGCA ACCCCCATCT  
56881 TCTACTGGGA TGAAGGCCGC TCCTGGGTCC TCACCCGATA CCACGACGTG TCGGCGGTGT  
56941 TCCGCGACGA ACGCTTCGCG GTCAGTCGAG AAGAATGGGA ATCGAGCGCG GAGTACTCGT  
10 57001 CGGCCATTCC CGAGCTCAGC GATATGAAGA AGTACGGATT GTTCGGGCTG CCGCCGGAGG  
57061 ATCAGCTCG GGTCCGCAAG CTCGTCAACC CATCGTTTAC GTCACGCGCG ATCGACCTGC  
57121 TCGCGGCCGA AATACAGCG ACCGTCGACC AGCTGCTCGA TGCTCGCTCC GGACAAGAGG  
57181 AGTTCGACGT TGTGCGGGAT TACGCGGAGG GAATCCCGAT GCGTGCGATC AGCGCTCTGT  
57241 TGAAGGTTCC GGCCGAGTGT GACGAGAAGT TCCGTGCTT CGGCTCGGCG ATCGCGCGCG  
15 57301 CGCTCGGCGT GGGTTTGGTG CCGCGGGTCC ATGAGGAGAC CAAGACCCTG GTCGCGTCCG  
57361 TCACCGAGGG GCTCGCGCTG CTCCATGGCG TCCTCGATGA GCGGCGCAGG AACCCTGCTG  
57421 AAAATGACGT CTTGACGATG CTGCTTCAGG CCGAGGCCGA CCGCAGCAGG CTGAGCACGA  
57481 AGGAGCTGGT CGCGCTCGTG GGTGCGATTA TCGCTGCTGG CACCGATACC ACGATCTACC  
57541 TTATCGCGTT CGCTGTGCTC AACCTGCTGC GGTGCGCCGA GCGCTCGAG CTGGTGAAGG  
20 57601 CCGAGCCCGG GCTCATGAGG AACGCGCTCG ATGAGGTGCT CCGCTTCGAC AATATCCTCA  
57661 GAATAGGAAC TGTGCGTTTC GCCAGGCAGG ACCTGGAGTA CTGCGGGGCA TCGATCAAGA  
57721 AAGGGGAGAT GGTCTTTCTC CTGATCCCGA GCGCCCTGAG AGATGGGACT GTATTCTCCA  
57781 GGCCAGACGT GTTTGATGTG CGACGGGACA CGAGCGCGAG CCTCGCGTAC GGTAGAGGCC  
57841 CCCATGTCTG CCGCGGGGTG TCCCTTGCTC GCCTCGAGGC GGAGATCGCC GTGGGCACCA  
25 57901 TCTTCCGTAG GTTCCCCGAG ATGAAGCTGA AAGAACTCC CGTGTTTGGA TACCACCCCG  
57961 CGTTCCGGAA CATCGAATCA CTCAACGTCA TCTTGAAGCC CTCAAAGCT GGATAACTCG  
58021 CCGGGGCGATC GCTTCCCGAA CCTCATTCTT TCATGATGCA ACTCGCGCGC GGGTGCTGTC  
58081 TGCCGCGGGT GCGATTGAT CCAGCGGACA AGCCCATTGT CAGCGCGCGA AGATCGAATC  
58141 CACGGCCCCG AGAAGAGCCC GATGGCGAGC CCGTCCGGGT AACGTCGGAA GAAGTGCCGG  
30 58201 GCGCGGCCCT GGGAGCGCAA AGCTCGCTCG CTCGCGCTCA GCGCGCCGCT TGCCATGTCC  
58261 GGCCCTGCAC CCGCACCAGG AGCCACCCG CCTGATGCA CGGCCTCACC GAGCGGCAGG  
58321 TTCTGCTCTC GCTCGTCGCC CTCGCGCTCG TCCTCCTGAC CGCGCGCGCC TTCGGCGAGC  
58381 TCGCGCGGCG GCTGCGCCAG CCGAGGTGC TCGGCGAGCT CTTGCGCGGC GTGGTGCTGG  
58441 GCGCGTCCGT CGTCGGCGCG CTCGCTCCTG GGTTCATCG AGTCCTCTT CAGGATCCGG  
35 58501 CGGTGCGGGG CGTGCTCTCC GGCATCTCCT GGATAGGCGC GCTCGTCTTG CTGCTCATGG  
58561 CCGGTATCGA GGTGATGTG AGCATCTTCT GCAAGGAGG CCGCCCCGGG GCGCTCTCGG  
58621 CGCTCGGCGC GATCGCGCCC CCGCTGCGCA CGCCGGGCCC GCTGGTGAG GCGATCGAGG  
58681 GCACGTTGAC GTGGGATCTC GACGTCTCGC CGCGACGCTC TGCGCAAGCC TGAGCCTCGG  
58741 CGCCTGCTCG TACACCTCGC CCGTGCTCGC TCCGCCCGCG GACATCCGGC CGCCCCCGC  
40 58801 GGCCAGCTC GAGCCGGACT CGCCGGATGA CGAGGCCGAC GAGGCGCTCC GCGGCTCCG  
58861 CGACGCGATC GCCGCTACT CCGAGGCCGT TCGGTGGGCG AAGGCGCTCG ACAAGGCACC  
58921 GCTGGAGAGC CTCGTGCGGC TCGCGATCGT GCGGCTGGGC GCGCGCCTTC ACCCCGCGGA  
58981 TTTGCGGCAC ACGACGGCCG GCGTCTCCCA GATCGCCGGC AGACTTCCCC AGAAAACGAA  
59041 TGCGGTCTGG TTCGATGTCG CCGCCCGGTA CGCGAGCTTC CGCGCGCGCA CGGAGACGC  
45 59101 GCTCCGCGAC GCGGCGTCCG CCACGGAGGC GCTCGCGGCC GGCCCGTACC GCGGATCGAG  
59161 CAGCGTGTC GGTGCCGTAG GGGAGTTTCG GGGGGAGGCG GCGCGCCTTC ACCCCGCGGA  
59221 CCGCGTACCC GCGTCCGACC AGCATTCTT GACCGCGCTG CCGCGAGCCG AGCGGGCGCT  
59281 CATCGCGCTC TACACCGCGT TCGCCCGTGA GGAGTGAGCC TCTCTCGGGC GCAGCCGAGC  
50 59341 GCGGCGGTGC CCGTTGTTCC CTCTTCGCAA CCATGACCGG AGCCCGCGCC GGTCCGCGCA  
59401 GCGGTAGCG CCGTTCGAGG CAGAGAGCGC TGGAGCGACA GGCGACGACC CGCCCGAGGG  
59461 TGTCGAACGG ATTGCCGAG CCCTCATTGC GGATCCCTTC CAGACACTCG TTCAGCGCCT  
59521 TGCGGTTCGAT GCGGCTGGG CACTCGCCGA AGGTCAGCTC GTCGCGCCAG TCGGATCGGA  
59581 TCTTGTTTGA GCACGATCC TTGCTCGAAT ACTCCCGGTC TTGTCCGATG TTGTTGCACC  
59641 GCGCCTCGCG GTGCGACCGC CGCGCCACGA TGCTATCGAC GCGGCTGCGC ACTGGCACC  
55 59701 GCGCCTCGCC TTGCGCGCCA CCGGGGTTT GCGCCTCCCC GCGTACCGC TTTTCCCGC  
59761 CGCACGCCGC CGCGAGCAGG CTCATTCCCG ACATCGAGAT CAGGCCACG ACCAGTTTCC  
59821 CAGCAATCTT TTGCATGGCT TCCCCTCCCT CACGACAGT CACATCAGAG ATTCTCCGCT  
59881 CCGCTCGTCG GTTCGACAGC CGGCGACGGC CACGAGCAGA ACCGTCCCCG ACCAGAACAG  
59941 CCGCATGCGG GTTTCTCGCA GCATGCCACG ACATCCTTGC GACTAGCGTG CCTCCGCTCG

5 60001 TGCCGAGATC GGCTGTCCTG TGCACGGGCA ATGTCCTGCG ATCGGCCGGG CAGGATCGAC  
60061 CGACACGGGC GCCGGGCTGG AGGTGCCGCC ACGGGCTCGA AATGCGCTGT GGCAGGCGCC  
60121 TCCATGCCCG CTGCCGGGAA CGCAGCGCCC GGCCAGCCTC GGGGCGACGC TCGAACGGG  
60181 AGATGCTCCC GGAGAGGCGC CGGGCACAGC CGAGCGCCGT CACCACCGTG CGCACTCGTG  
60241 AGCGCTAGCT CCTCGGCATA GAAGAGACCG TCACTCCCGG TCCGTGTAGG CGATCGTGCT  
60301 GATCAGCGCG TCCTCCGCCT GACGCGAGTC GAGCCGGGTA TGCTGCACGA CGATGGGCAC  
60361 GTCCGATTTC ATCAGCTGG CATAGTCCGT ATCGCGCGGG ATCGGCTCGG GGTCCGTCAG  
60421 ATCGTTGAAC CGGACGTGCC GGGTGCGCCT CGCTGGAACG GTCACCCGGT ACGGCCCGGC  
10 60481 GGGGTCGCGG TCGCTGAAGT AGACGGTGAT GGCGACCTGC GCGTCCCGGT CCGACGCATT  
60541 CAACAGGCAG GCCGTCTCAT GGCTCGTCAT CTGCGGCTCA GGTCCGTTGC TCCGGCCTGG  
60601 GATGTAGCCC TCTGCGATTG CCCAGCGCGT CCGCCCGATC GGCTTGTCCA TGTGTCTCTC  
60661 CTCCTGGCTC CTCTTTGGCA GCCTCCCTCT GCTGTCCAGG TGCGACGGCC TCTTCGCTCG  
60721 ACGCGCTCGG GGCTCCATGG CTGAGAATCC TCGCCGAGCG CTCCTTGCCG ACCGGCGCGC  
60781 TGAGCGCCGA CGGGCCTTGA AAGCACGCGA CCGGACACGG GATGCCGGCG CGACGAGGCC  
15 60841 GCCCCGCGTC TGATCCCGAT CGTGGCATCA CGACGTCCGC CGACGCCTCG GCAGGCCGGC  
60901 GTGAGCGCTG CGCGGTCATG GTCGTCTCTG CGTACCGCC ACCCGCCGAT TCACATCCCA  
60961 CCGCGGCACG ACGCTTGCTC AAACCGCGAC GACACGCGCG GCGGCTGTG GTACCGGCCA  
61021 GCCCGGACGC GAGGCCGAGC AGGGACAGTG GGTCCGCGGT GAAGCAGAGA GGCGATCGAG  
20 61081 GTGGTGAGAT GAAACACGTT GACACGGGCC GACGAGTCGG CCGCCGGATA GGGCTCACGC  
61141 TCGGTCTCCT CGCGAGCATG GCGCTCGCGG GCTGCGGCGG CCCGAGCGAG AAGACCGTGC  
61201 AGGGCACGCG GCTCGCGCCC GCGCCCGATG CGCACGTCAC CGCCGACGTC GACGCCGACG  
61261 CCGCGACCAC GCGGCTGGCG GTGGACGTCG TTCACCTCTC GCCGCCGAG CCGATCGAGG  
61321 CCGCGAGCGA GCGGTTCTGT CGACTACAAC CTGAGTCCG GTCGAACTC CGAGTCCCCG TGGTACGGG  
25 61381 TCGGAGTGCT CGAGAGTCTC CTGAGTCCG GATGCCAGC GAAGAGGCAA GCTGGCCGAG ACGACCGTGC  
61441 CGCATGCCAA CTTCGAGCTG CTCATCACCG TCGAGAAGCA GAGCAGCCCT CAGTCGCCAT  
61501 CGTCTGCCGC CGTCATCGGG CCGACGTCCG TCGGGTAACA TCGCGCTATC AGCAGCGCTG  
61561 AGCCCGCCAG CATGCCCCAG AGCCCTGCCT CGATCGCTTT CCCCATCATC CGTGCGCACT  
61621 CCTCCAGCGA CCGCCGCGTC AAAGCAACCG CCGTGCCGGC GCGGCTCTAC GTGCGCGACA  
30 61681 GGAGAGCGTC CTAGCGCGGC CTGCGCATCG CTGGAAGGAT CGGCGGAGCA TGGAGAAAGA  
61741 ATCGAGGATC GCGATCTACG GCGCGTTCG CGCCAACGTG GCGATCGCGG CGGTCAAGTT  
61801 CATCGCCGCC GCCGTGACCG GCAGCTCTGC GATGCTCTCC GAGGGCGTGC ACTCCCTCGT  
61861 CGATACCGCA GACGGGCTCC TCCTCCTGCT CCGCAAGCAC CGGAGCGCCC GCCCGCCGA  
61921 CGCCGAGCAT CCGTTCCGGC ACGGCAAGGA GCTCTATTTT TGGACGCTGA TCGTCGCCAT  
35 61981 CATGATCTTC GCCGCGGGCG GCGGCGTCTC GATCTACGAA GGGATCTTGC ACCTCTTGCA  
62041 CCGCGCTCG ATCGAGGATC CGACGTGGAA CTACGTTGTC CTCGGCGCAG CGGCCGTCTT  
62101 CGAGGGGACG TCGTTCGCCA TCTCGATCCA CGAGTTCAAG AAGAAAGACG GACAGGGCTA  
62161 CGTCGCGGCG ATGCGGTCCA GCAAGGACCC GACGACGTTT ACGATCGTCC TGGAGGATTC  
62221 CGCGGCGCTC GCCGGGCTCG CCATCGCCTT CCTCGGCGTC TGGCTTGGGC ACCGCTGGG  
40 62281 AAACCCCTAC CTCGACGGCG CCGCGTCGAT CGGCATCGGC CTCGTGCTCG CCGCGGTTCG  
62341 GGTCTTCCTC GCCAGCCAGA GCCGTGGACT CCTCGTAGGG GAGAGCGCGG ACAGGGAGCT  
62401 CCTCGCGCG ATCCGCGCGC TCGCCAGCGC AGATCCTGGC GTGTGCGCGG TGGGGCGGGC  
62461 CCTGACGATG CACTTCGGTC CGCACGAAGT CCTGGTCTGT CTGCGCATCG AGTTCGACGC  
62521 CGCGCTCACG GCGTCCGGGG TCGCGGAGGC GATCGAGCGA ATCGAGACAC GGATACGGAG  
45 62581 CGAGCGACCC GACGTGAAGC ACATCTACGT CGAGGCCAGG TCGCTCCACC AGCGCGCGAG  
62641 GCGGTGACGC GCCGTGGAGA GACCGCTCGC GGCTCCGCC ATCCTCCGCG GCGCCCGGGC  
62701 TCGGGTAGCC CTCGACGAG GCGCGCGCTG GCGGGCAAAC CGTGAAGACG TCGTCTTCG  
62761 ACGCGAGGTA CGCTGGTTGC AAGTTGTAC GCCGTATCGC GAGGTCCGGG AGCGCCGGAG  
50 62821 CCGGGGCGGT CCGGGCGCAC GAAGGCCCGG CGAGCGCGGG CTTCGAGGGG GCGACGTCAT  
62881 GAGGAAGGGC AGGGCGCATG GGGCGATGCT CCGCGGGCGA GAGGACGGCT GCGTCGCGG  
62941 CCTCCCCGGC GCCGGCGCGC TTCGCGCCGC GCTCCAGCGC GGTCTGCTCG GCGATCTCGC  
63001 CCGGCGCCGG CTCATCGCCG CCGTGTCCCT CACCGGCGGC GCCAGCATGG CCGTCTCTC  
63061 GCTGTTCCAG CTCGGGATCA TCGAGCACCT GCCCGATCCT CCGCTTCCAG GGTTCGATT  
55 63121 GGCCAAGGTG ACGAGCTCCG ATATCGCGTT CCGGCTCAGG ATGCCGGAGC CGCCGCTCGC  
63181 GCTCACCAGC TTCGCGTCCA ACCTGGCGCT GGCTGGCTGG GGAGGCGCGG AGCGCGCCAG  
63241 GAACACCCCC TGGATCCCCG TCGCCGTGGC GGCCAAGGCG GCCGTCGAGG CGCGCGTGT  
63301 CGGATGGCTC CTCGTCCAGA TCGACGGCG GGAGAGGGCC TGGTGGCGCT ACTGCTGGT  
63361 CGCCATGGCG GCCAACATGG CCGTGTTCGC GCTCTCGCTC CCGGAAGGGT GGGCGCGCT  
63421 GAGGAAGGCG CGAGCGCGCT CGTGACAGG CCGTGCGGGC GCCCGGCCA TCGGAGGCCG  
63481 GCGTGACCC GCTCCGTAC GCCCGGGCC GCGCGCGGT GAGCTGCCG GGACAGGGC

5 63541 CGTACCGTGG ACCCCGACAG CGCCGCGTCG ACGGACATCC CCGGCGGCTC GCGCGGCGCG  
63601 GCCGGCGCAA CTCCGGCCCG CCGCCGGGCA TCGACATCTC CCGCGAGCAA GGGCACTCCG  
63661 CTCCTGCCCG CGTCCGCGAA CGATGGCTGC GCTGTTTCCA CCCTGGAGCA ACTCCGTTTA  
63721 CCGCGTGGCG CTCGTGCGGC TCATCGCCTC GCGGGGCGGC GCCATCCTCG CGCTCATGAT  
63781 CTACGTCCGC ACGCCGTGGA AGCGATACCA GTTCGAGCCC GTCGATCAGC CGGTGCAGTT  
63841 CGATCACCGC CATCACGTGC AGGACGATGG CATCGATTGC GTCTACTGCC ACACCACGGT  
63901 GACCCGCTCG CCGACGGCGG GGATGCCGCC GACGGCCACG TGCATGGGGT GCCACAGCCA  
63961 GATCTGGAAT CAGAGCGTCA TGCTCGAGCC CGTGCGGCGG AGCTGGTTCT CCGGCATGCC  
10 64021 GATCCCGTGG AACCGGGTGA ACTCCGTGCC CGACTTCGTT TATTTCAACC ACGCGATTCA  
64081 CGTGAACAAG GCGGTGGGCT GCGTGAGCTG CCACGGGCGC GTGGACGAGA TGGCGGCCGT  
64141 CTACAAGGTG GCGCCGATGA CGATGGGCTG GTGCCGAGAG TGCCATCGCC TGCCGGAGCC  
64201 GCACCTGCGC CCGCTCTCCG CGATACCCGA CATGCGCTGG GACCCGGGGG AACGGAGGGA  
64261 CCGGCTCGGG GCGAAGCTCG CGGAGGAGTA CCGGGTCCGG CCGCTCACGC ACTGCACAGC  
64321 GTGCCATCGA TGAACGATGA ACAGGGGATC TCCGTGAAAG ACGCAGATGA GATGAAGGAA  
15 64381 TGGTGGCTAG AAGCGCTCGG GCCGGCGGGA GAGCGCGCGT CCTACAGGCT GCTGGCGCCG  
64441 CTCATCGAGA GCGCGGAGCT CCGCGCGCTC GCCGCGGGCG AACCGCCCCG GGGCGTGGAC  
64501 GAGCCGGCGG GCGTCAGCCG CCGCGCGCTG CTCAGCTGC TCGGCGCGAG CATGGCGCTC  
64561 GCCGGCGTGC CCGGCTGCAC CCGCGATGAG CCCGAGAAGA TCCTGCCGTA CAACGAGACC  
64621 CCGCGCGCGC TCGTGCCGGG TCTCTCCAG TCCTACGCGA CGAGCATGGT GCTCGACGGG  
20 64681 TATGCCATGG GCCTCCTCGC CAAGAGCTAC GCGGGGCGGC CCATCAAGAT CGAGGGCAAC  
64741 CCCGCGCACC CCGCGAGCCT CCGCGCGACC GCGGTCCACG AGCAGGCCTC GATCCTCTCG  
64801 CTGTACGACC CGTACCGCGC GCGCGCGCCG ACGCGCGCGG GCCAGGTGCG GTCGTGGGAG  
64861 GCGCTCTCCG CCGCTTCCG CCGCGACCGC GAGGACGGCG GCGCTGGCCT CCGCTTCGTC  
64921 CTCAGCCCCA CGAGCTCGCC CTCATCGCC GCGCTGATCG AGCGCGTCCG GCGCAGGTTT  
25 64981 CCGGGCGCGC GGTTCACCTT CTGGTCGCCG GTCCACGCGG AGCAAGCGCT CGAAGGCGCG  
65041 CCGGCGGCGC TCGGCCTCAG GCTCTTGCTT CAGCTCGACT TCGACCAAGT CGAGGTGATC  
65101 CTCGCCCTGG ACGCGGACTT CCTCGCGGAC ATGCCGTTCA GCGTGCGCTA TGCGCGCGAC  
65161 TTCGCCGCGC GCCGCCGACC CCGGAGCCCG GCGGCGGCCA TGAACCGCCT CTACGTGCGG  
30 65221 GAGGCGATGT TCACGCCAC GGGGACGCTC GCCGACCACC GGCTCCGCGT GCGGCCCGCC  
65281 GAGGTCGCGC GCGTCGCGG CGGCTCGCG CCGGAGCTCG TGCACGGCCT CCGCCTGCGC  
65341 CCGCGCGGGA TCACGGACGC CGACGCCGCC GCGCTGCGCG CGCTCCGCC CCGCCCGCGC  
65401 GAGGGGACAG GCGCCTTCGT CCGGGCGCTC GCGCGCGATC TCGCGCGCGC GGGGGGCGCC  
65461 GCGCTGCGCG TCGTCGCGGA CCGCCAGCCG CCCATCGTCC ACGCCCTCGG GCACGTCATC  
35 65521 AACGCCGCGC TCCGACGCCG GCGGCGCTGG ATGGTCGATC CTGTGCTGAT CGACGCGGGC  
65581 CCCTCCACGC AGGGCTTCTC CGAGCTCGTC GCGGAGCTCG GCGCGGGCGC GGTCGACACC  
65641 TGATCCTCCT CGACGTGAAC CCGGTGACG CCGGCGCGCTA CGACGACGAG ACCGCCCGCG  
65701 TCCTCGCGCG CGTGCCACG AGCTTGAAG CCGGGCTCTA CGACGACGAG TCCGCCCGCG  
65761 CTTGCACGTG GTTCGTGCGG ACCCGGCATT ACCTCGAGTC GTGGGGGGAC GCGCGGGCGT  
40 65821 ACGACGGGAC GGTCTCGTTC GTGCAACCC TCGTCCGGCC GCTGTTTCGAC GGCCGGGCGG  
65881 TGCCCAGACT GCTCGCCGTC TTCGCGGGGG ACGAGCGCCC GGATCCCCGG CTGCTGCTGC  
65941 GCGAGCACTG GCGCGGCGCG CCGGAGAGG CCGATTTCGA GGCCTTCTGG GCGGAGGCAT  
66001 TGAAGCGCGG CTTCTCCCT GACAGCGCCC GCGCGAGGCA GACACCGGAT CTCGCGCCGG  
66061 CCGACCTCGC CAAGGAGCTC GCGCGGCTCG CCGCGCGCGC GCGGCGGCGC GCGGCGCGC  
45 66121 TCGACGTGGC GTTCTCAGG TCGCCGTCGG TCCACGACGG CAGGTTCCGC AACAAACCCCT  
66181 GGCTGCAAGA GCTCCCGCGG CCGATACCA GGCTCACCTG GGGCAACGCC GCCATGATGA  
66241 GCGCGGCGAC CCGGCGCGCG CTCGGCGTCC AGCGCGGCGA TGTGCTCGAG CTCGCGCTGC  
66301 CCGGCGGTAC GATCGAGATC CCGGCCGTCG TCGTCCGCGG GCACGCCGAG GACGTGATCA  
66361 GCGTCGACCT CCGCTACGGG CCGGACGCCG CCGAGGAGGT CCGCGCGGG GTGGCGTGT  
50 66421 CCGCGTATCG GATCCGCCCG TCCGACGCGC GGTGGTTTCG GGGGGGCTC TCCGTGAGGA  
66481 AGACGGGCGC CACGGCCGCG CTCGCGCTGG CTCAGATCGA GCTGTCCCAG CACGACCGTC  
66541 CCATCGCGCT CCGGAGGACG CTGCCGAGT ACCGTGAACA GCCCGGTTTC GCGGAGGAGC  
66601 ACAAGGGGCC GGTCCGCTCG ATCCTGCCGG AGGTGAGTA CACCGGCGCG CAATGGGCGA  
66661 TGTCCATCGA CATGTCGATC TGACCCGGGT GCTCCTCGTG CGTCTGGGCC TGTCAGGCCG  
55 66721 AGAACAACGT CCTCGTCGTC GCGAAGGAG AGGTGATGCA CCGCGCGGAG ATGCAAGTGGT  
66781 TGCGGATCGA TCAGTACTTC GAGGGTGGAG GCGACGAGGT GAGCGTCGT AACCAGCCGA  
66841 TGCTCTGCCA GCACTGCGAG AAGGCGCCGT GCGAGTACGT CTGTCCGGTG AACGCGACGG  
66901 TCCACAGCCC CGATGGCCTC AACGAGATGA TCTACAACCG ATGCATCGGG ACGCGCTTTT  
66961 GCTCCAACAA CTGTCCGTAC AAGATCCGGC GGTTCATTT CTTCGACTAC AATGCCACG  
67021 TCCCGTACAA CCGCGGCTC CGCAGGCTCC AGCGCAACCC GGACGTCACC GTCCGCGCCC

5 67081 GCGGCGTCAT GGAGAAATGC ACGTACTGCG TGCAGCGGAT CCGAGAGGCG GACATCCGCG  
67141 CGCAGATCGA GCGGCGGCCG CTCCGGCCGG GCGAGGTGGT CACCGCCTGC CAGCAGGCCCT  
67201 GTCCGACCGG CGCGATCCAG TTCGGGTCGC TGGATCACGC GGATACAAAG ATGGTCGCGT  
67261 GGCGCAGGGA GCCGCGCGCG TACGCCGTGC TCCACGACCT CGGCACCCGG CCGCGGACGG  
67321 AGTACCTCGC CAAGATCGAG AAACCCGAACC CGGGGCTCGG GGCGGAGGGC GCCGAGAGGC  
67381 GACCCGGAGC CCCGAGCGTC AAACCCGCGC TCGGGGCGGA GGGCGCCGAG AGGCGACCCG  
67441 GAGCCCCGAG CGTCAAACCG GAGATTGAAT GAGCCATGGC GGGCCCCGCTC ATCCTGGACG  
10 67501 CACCGACCGA CGATCAGCTG TCGAAGCAGC TCCTCGAGCC GGTATGGAAG CCGCGCTCCC  
67561 GGCTCGGCTG GATGCTCGCG TTCGGGCTCG CGCTCGGCGG CACGGGCTTG CTCTTCTCG  
67621 CGATCACCTA CACCGTCTCT ACCGGGATCG GCGTGTGGGG CAACAACATC CCGGTCGCTT  
67681 GGGCCTTCGC GATACCAAC TTCGTCTGGT GGATCGGGAT CCGCCACGCC GGGACGTTCA  
67741 TCTCCGCGAT CCTCCTCTG CTGAGCAGA AGTGGCGGAC GAGCATCAAC CGCTTCGCCG  
67801 AGGCGATGAC GCTCTTCGCG GTCGTCCAGG CCGGCTCTT TCCGGTCTCT CACCTCGGCC  
15 67861 GCCCCTGGTT CGCCTACTGG ATCTTCCCGT ACCCGCGGAC GATGCAGGTG TGGCCGAGT  
67921 TCCGGAGCGC GCTGCCGTGG GACGCCGCG CGATCGCGAC CTACTTCACG GTGTCTCTCC  
67981 TGTTCGGTA CATGGGCCTC GTCCCGATC TGGCGGCGCT GCGCGACCAC GCCCGGGGCC  
68041 GCGTCCGCGG GGTGATCTAC GGGCTCATGT CGTTCCGGCTG GCACGGCGCG GCCGACCACT  
20 68101 TCCGCGATTA CCGGGTGCTG TACGGGCTCG TCGCGGGGCT CGCGACGCCCT CTGTCGTCT  
68161 CGGTGCACTC GATCGTGAGC AGCGATTTTC CGATCGCCCT GGTGCCCGG TGGCACTCGA  
68221 CGCTCTTTCC GCCGTTCTTC GTCGCGGGCG CGATCTTCTC CCGGTTTCGCG ATGGTGCTCA  
68281 CGGTGCTCAT CCCGGTGCGG CGGATCTACG GGCTCCATAA CGTCGTGACC GCGCGCCACC  
68341 TCGACGATCT CGCGAAGATG ACCTCTGTA CCGGCTGGAT CGTCATCCTC TCGTACATCA  
68401 TCGAGAACTT CCTCGCCTGG TACAGCGGCT CGGCGTACGA GATGCATCAG TTTTTCAGA  
25 68461 CGCGCCTGCA CCGCCCCAAC AGCGCCGCT ACTGGGCCCA GCACGTCTGC AACGTGCTCG  
68521 TCATCCAGCT CCTCTGGAGC GAGCGGATCC GGACGAGCCC CGTCGCGCTC TGGCTCATCT  
68581 CCCTCCTGGT CAACGTGCGG ATGTGGAGCG AGCGGTTTAC GCTCATCGTG ATGTCTCTCG  
68641 AGCAAGAGTT CCTCCCGTCC AAGTGGCAG GCTACAGCCC GACGTGGGTG GACTGGAGCC  
68701 TCTTCATCGG GTCAGGCGGC TTCTTCATGC TCCTGTCTCT GAGCTTTTTG CGCGTCTTTC  
30 68761 CGTTCATCCC CGTCGCGGAG GTCAAGGAGC TCAACCATGA AGAGCTGGAG AAGGCTCGGG  
68821 GCGAGGGGGG CCGGTGATGG AGACCGGAAT GCTCGGCGAG TTCGATGACC CGGAGGCGAT  
68881 GCTCCATGCG ATCCGAGAGC TCAGGCGGCG CCGCTACCGC CCGGTGGAAG CGTTCACGCC  
68941 CTATCCGGTG AAGGGGCTCG ACAGGCGCT CGGCCTCCCG CGCTCGAACC TCAACCGGAT  
69001 GGTGCTGCCC TTCGCGATCC TGGGGGTCTG GGGCGGCTAC TTCGTCCAGT GGTTCGCAA  
35 69061 CGCTTTCCAC TATCCGCTGA ACGTGGGCGG GCGCCCGCTG AACTCGGCGC CGGCGTTCAT  
69121 CCCGATCAGG TTCGAGATGG GGTGCTCTC CACCTCGATC TTCGGCGTGC TCATCGGCTT  
69181 TTACCTGACG AGGCTGCCGA GGCTCTACCT CCCGCTTTC GACGCCCCGG GCTTCGAGCG  
69241 CGTCACGCTG GATCGGTTTC TGGTCGGGCT CGACGACACG GAACCTTCCT TCTCGAGCGC  
69301 CCAGGCGGAG CGCGACCTCC TCGCGCTCGG CGCCCGGCGC GTCGTCTGTC CGAGGAGCGC  
40 69361 CGAGGAGCCA TGAGGGCCGG CGCCCGGCT CGCCCTCTCG GGCGCGCGCT CGCGCCGTTT  
69421 GCCCTCGTCC TGCTCGCCGG GTGCCGCGAG AAGGTGCTGC CCGAGCCGGA CTTGAGCGG  
69481 ATGATCCGCC AGGAGAAATA CGGACTTGG GAGCCGTGCG AGCACTTCGA CGACGGCCGC  
69541 GCGATGCAGC ACCCGCCCGA GGGGACCCTG GCGCGCGGGC GCGTACCAG GCGCCCGG  
69601 TATCTCCAGG GCGTCTCTGA CCGGGCGTAC GTCACGGAGG TGCCGCTCTT GCTCACGGTC  
45 69661 GAGCTCGTGC AGCGCGGCCG GCAGCGCTTC GAGACCTTCT GCGCGCCGTG CCACGGGATC  
69721 CTCGGCGACG GCAGCTCGCG CGTGGCGACG AACATGACGC TGGCCCGCC CCCGTCGCTC  
69781 ATCGGACCCG AGGCGCGGAG CTTCCCGCG GGCAGGATCT ACCAGGTCAT CATCGAGGGC  
69841 TACGGCCTGA TGCCGCGCTA CTCGGACGAT CTGCCGACA TCGAAGAGCG CTGGGCGGTC  
50 69901 GTCGCTACG TGAAGGCGCT TCAGCTGAGC CGCGGAGTGG CCGCGGGCGC CCTCCCGCCA  
69961 GCGCTCCGCG GCCGGGCGA GCAGGAGCTG CGATGAACAG GGATGCCATC GAGTACAAG  
70021 GCGGCGCGAC GATCGCGGCC TCGCTCGCGA TCGCGGCGCT CGGCGCGGTC GCCGCGATCG  
70081 TCGGCGGCTT CGTCGATCTC CGCCGTTTCT TCTTCTCGTA CCTCGCCGCG TGGTCGTTCC  
70141 CGGTGTTTTCT GTCCGTGGGC GCGCTCGTCA CGCTCCTCAC CTGCAACGCC ATGCGCGCGG  
70201 GCTGGCCAC GCGGTGCGC CGCCTCTCG AGACGATGGT GCGCGCGCTG CCTCTGCTCG  
70261 CGGCGCTCTC CGCGCCGATC CTGGTCGGCC TGGACACGCT GTATCCGTGG ATGACCCCG  
55 70321 AGCGGATCGC CGGCGAGCAC GCGCGGCGCA TCCTCGAGCA CAGGGCGCCC TACTTCAATC  
70381 CAGGCTTCTT CGTCGTGCGC TCGGCGATCT ACTTCGCGAT CTGGATCGCC GTCGCCCTCG  
70441 TGCTCCGCG GCGATCGTTC GCGCAGGACC GTGAGCCGAG GGCCGACGTC AAGGACGCGA  
70501 TGTATGGCCT GAGCGGCGCC ATGCTGCCGG TCGTGGCGAT CACGATCGTC TTCTCGTCTG  
70561 TCGACTGGCT CATGTCCCTC GACGCGACCT GGTACTCGAC GATGTTCCCG GTCTACGTGT

70621 TCGCGAGCGC CTTCGTGACC GCCGTCGGCG CGCTCACGGT CCTCTCGTAT GCCGCGCAGA  
 70681 CGTCCGGCTA CCTCGCGAGG CTGAACGACT CGCACTATTA CGCGCTCGGG CGGCTGCTCC  
 70741 TCGCGTTCAC GATATTCTGG GCCTATGCGG CCTATTTCCA GTTCATGTTG ATCTGGATCG  
 5 70801 CGAACAGCC CGATGAGGTC GCCTTCTTCC TCGACCGCTG GGAAGGGCCC TGGCGGCCGA  
 70861 CCTCCGTGCT CGTCGTCTC ACGCCGTTCC TCGTCCCGTT CCTGATCCTG ATGTCGTACG  
 70921 CGATCAAGCG GCGCCCGCGC CAGCTCTCGT GGATGGCGCT CTGGGTCGTC GTCTCCGGCT  
 70981 ACATCGACTT TCACTGGCTC GTGGTGCCGG CGACAGGGCG CCACGGGTTT GCCTATCACT  
 71041 GGCTCGACCT CGCGACCCTG TCGCTCGTGG GCGGCCCTCT GACCGCGTTC GCCGCGTGGC  
 10 71101 GGTGCGGAGG GCGGCCGGTG GTCCCGGTCC ACGACCCGCG GCTCGAAGAG GCCTTTGCGT  
 71161 ACCGGAGCAT ATGATGTTCC GTTTCCGTCA CAGCGAGGTT CGCCAGGAGG AGGACACGCT  
 71221 CCCCTGGGGG CGCGTGATCC TCGCGTTCGC CGTCGTGCTC GCGATCGGCG GCGCGCTGAC  
 71281 GCTCTGGGCG TGGCTCGCGA TGCGGGCCCG CGAGGCGGAT CTGCGGCCCT CCCTCGCGTT  
 71341 CCCCAGAGAAG GATCTCGGGC CGCGGCGCGA GGTGCGCATG GTCCAGCAGT CGCTGTTCTGA  
 15 71401 CGAGGCGCGC CTGGGCCAGC AGCTCGTCGA CGCGCAGCGC GCGGAGCTCC GCCGTTCTCG  
 71461 CGTCGTGATC CGGGAGAGGG GCATCGTGAG CATCCCGATC GACGACGCGA TCGAGTCAAT  
 71521 GGTGGCGGGG GCGCGCGGAT GAGCCGGGCC GTCGCCGTGG CCCTCCTGCT GGCAGCCGGC  
 71581 CTCGTGTGCG GCCCGGGCGC CGCGTCCGAG CCCGAGCGCG CGCGCCCCGC GCTGGGCCCG  
 71641 TCCGCGGGCG ACGCCGCGCC GGCGAGCGAC GGCTCCGGCG CGGAGGAGCC GCCCGAAGGC  
 20 71701 GCCTTCCTGG AGCCACGCG CGGGGTGGAC ATCGAGGAGC GCCTCGGCCG CCCGGTGGAC  
 71761 CGCGAGCTCG CCTTCACCGA CATGGACGGG CGGCGGGTGC GCCTCGGCCA CTACTTCGCG  
 71821 GACGGCAAGC CCCTCCTCCT CGTCTCGCG TACTACCGGT GTCCCGCGCT GTGCGGCCTC  
 71881 GTGCTGCGCG GCGCCGTCGA GGGGCTGAAG CTCCTCCCGT ACCGGCTCGG CGAGCAGTTC  
 71941 CACGCGCTCA CGGTCAGCTT CGACCCGCGC GAGCGCCCGG CGGCCGDD

25

### Example 2

#### Construction of a *Myxococcus xanthus* Expression Vector

The DNA providing the integration and attachment function of phage Mx8 was  
 inserted into commercially available pACYC184 (New England Biolabs). An ~2360 bp  
 MfeI-SmaI from plasmid pPLH343, described in Salmi *et al.*, Feb. 1998, J. Bact. 180(3):  
 30 614-621, was isolated and ligated to the large EcoRI-XmnI restriction fragment of plasmid  
 pACYC184. The circular DNA thus formed was ~6 kb in size and called plasmid  
 pKOS35-77.

Plasmid pKOS35-77 serves as a convenient plasmid for expressing recombinant  
 PKS genes of the invention under the control of the epothilone PKS gene promoter. In one  
 35 illustrative embodiment, the entire epothilone PKS gene with its homologous promoter is  
 inserted in one or more fragments into the plasmid to yield an expression vector of the  
 invention.

The present invention also provides expression vectors in which the recombinant  
 PKS genes of the invention are under the control of a *Myxococcus xanthus* promoter. To  
 40 construct an illustrative vector, the promoter of the *pilA* gene of *M. xanthus* was isolated  
 as a PCR amplification product. Plasmid pSWU357, which comprises the *pilA* gene  
 promoter and is described in Wu and Kaiser, Dec. 1997, J. Bact. 179(24):7748-7758, was  
 mixed with PCR primers Seq1 and Mxpil1 primers:

Seq1: 5'-AGCGGATAACAATTTACACAGGAAACAGC-3'; and

Mxpil1: 5'-TTAATTAAGAGAAGGTTGCAACGGGGGGC-3',

and amplified using standard PCR conditions to yield an ~800 bp fragment. This fragment was cleaved with restriction enzyme KpnI and ligated to the large KpnI-EcoRV restriction  
5 fragment of commercially available plasmid pLitmus 28 (New England Biolabs). The resulting circular DNA was designated plasmid pKOS35-71B.

The promoter of the *pilA* gene from plasmid pKOS35-71B was isolated as an ~800 bp EcoRV-SnaBI restriction fragment and ligated with the large MscI restriction fragment of plasmid pKOS35-77 to yield a circular DNA ~6.8 kb in size. Because the ~800 bp  
10 fragment could be inserted in either one of two orientations, the ligation produced two plasmids of the same size, which were designated as plasmids pKOS35-82.1 and pKOS35-82.2. Restriction site and function maps of these plasmids are presented in Figure 3.

Plasmids pKOS35-82.1 and pKOS35-82.2 serve as convenient starting materials for the vectors of the invention in which a recombinant PKS gene is placed under the  
15 control of the *Myxococcus xanthus pilA* gene promoter. These plasmids comprise a single PacI restriction enzyme recognition sequence placed immediately downstream of the transcription start site of the promoter. In one illustrative embodiment, the entire epothilone PKS gene without its homologous promoter is inserted in one or more fragments into the plasmids at the PacI site to yield expression vectors of the invention.

20 The sequence of the *pilA* promoter in these plasmids is shown below.

CGACGCAGGTGAAGCTGCTTCGTGTGCTCCAGGAGCGGAAGGTGAAGCCGGTCGGCAGCGCCGCGGAGATTC  
CCTTCCAGGCGCGTGTTCATCGCGGCAACGAACCGCGGCTCGAAGCCGAAGTAAAGGCCGACGCTTTCGTG  
AGGACCTCTTCTACCGGCTCAACGTATCAGCTTGGAGCTGCCCTCCACTGCGCGAGCGTTCCGGCGACGTGT  
25 CGTTGCTGGCGAACTACTTCTGTCCAGACTGTCCGAGGAGTTGGGGCGACCCGGTCTGCGTTTCTCCCCCG  
AGACACTGGGGCTATTGGAGCGCTATCCCTTCCCAGGCAACGTGCGGCAGCTGCAGAACATGGTGGAGCGGG  
CCGCGACCTGTCCGATTACAGACTCCTGGGGCCCTCCACGCTTCCACCCGAGTGCAGGGCGATACAGACC  
CCGCCGTGCGTCCCGTGGAGGGCAGTGAGCCAGGGCTGGTGGCGGGCTTCAACCTGGAGCGGCATCTCGACG  
ACAGCGAGCGGCGCTATCTCGTCGCGCGCATGAAGCAGGCCGGGGCGTGAAGACCCGTGCTGCGGAGTTGC  
TGGGCCTTTCGTTCGTTCCGTTACCGCTACCGGTTGGCCAAGCATGGGCTGACGGATGACTTGGAGCCCGGA  
30 GCGCTTCGGATGCGTAGGCTGATCGACAGTTATCGTCAGCGTCACTGCCGAATTTGTGACGCCCTGGACCCA  
TCCTCGCGGAGGGGATTGTTCCAAGCCTTGAGAATTGGGGGGCTTGGAGTGCACCTGGGTGGCATGCGT  
AGTGCTAATCCCATCCGCGGGCGCAGTGCCCCCGTTGCAACCTTCTCTTAATTAA

To make the recombinant *Myxococcus xanthus* host cells of the invention,  
*M. xanthus* cells are grown in CYE media (Campos and Zusman, 1975, Regulation of  
35 development in *Myxococcus xanthus*: effect of 3': 5'-cyclic AMP, ADP, and nutrition, Proc. Natl. Acad. Sci. USA 72: 518-522) to a Klett of 100 at 30°C at 300 rpm. The remainder of the protocol is conducted at 25°C unless otherwise indicated. The cells are then pelleted by centrifugation (8000 rpm for 10 min. in an SS34 or SA600 rotor) and

resuspended in deionized water. The cells are again pelleted and resuspended in 1/100th of the original volume.

DNA (one to two  $\mu\text{L}$ ) is electroporated into the cells in a 0.1 cm cuvette at room temperature at 400 ohm, 25  $\mu\text{FD}$ , 0.65 V with a time constant in the range of 8.8 - 9.4. The DNA should be free of salts and so should be resuspended in distilled and deionized water or dialyzed on a 0.025  $\mu\text{m}$  Type VS membrane (Millipore). For low efficiency electroporations, spot dialyze the DNA, and allow outgrowth in CYE. Immediately after electroporation, add 1 mL of CYE, and pool the cells in the cuvette with an additional 1.5 mL of CYE previously added to a 50 mL Erlenmeyer flask (total volume 2.5 ml). Allow the cells to grow for four to eight hours (or overnight) at 30 to 32°C at 300 rpm to allow for expression of the selectable marker. Then, plate the cells in CYE soft agar on plates with selection. If kanamycin is the selectable marker, then typical yields are  $10^3$  to  $10^5$  per  $\mu\text{g}$  of DNA. If streptomycin is the selectable marker, then it must be included in the top agar, because it binds agar.

With this procedure, the recombinant DNA expression vectors of the invention are electroporated into *Myxococcus* host cells that express recombinant PKSs of the invention and produce the epothilone, epothilone derivatives, and other novel polyketides encoded thereby.

20

### Example 3

#### Construction of a Bacterial Artificial Chromosome (BAC) for Expression of Epothilone in *Myxococcus xanthus*

To express the epothilone PKS and modification enzyme genes in a heterologous host to produce epothilones by fermentation, *Myxococcus xanthus*, which is closely related to *Sorangium cellulosum* and for which a number of cloning vectors are available, can also be employed in accordance with the methods of the invention. Because both *M. xanthus* and *S. cellulosum* are myxobacteria, it is expected that they share common elements of gene expression, translational control, and post translational modification (if any), thereby enhancing the likelihood that the epo genes from *S. cellulosum* can be expressed to produce epothilone in *M. xanthus*. Secondly, *M. xanthus* has been developed for gene cloning and expression. DNA can be introduced by electroporation, and a number of vectors and genetic markers are available for the introduction of foreign DNA, including those that permit its stable insertion into the chromosome. Finally, *M. xanthus* can be

grown with relative ease in complex media in fermentors and can be subjected to manipulations to increase gene expression, if required.

To introduce the epothilone gene cluster into *Myxococcus xanthus*, one can build the epothilone cluster into the chromosome by using cosmids of the invention and  
5 homologous recombination to assemble the complete gene cluster. Alternatively, the complete epothilone gene cluster can be cloned on a bacterial artificial chromosome (BAC) and then moved into *M. xanthus* for integration into the chromosome.

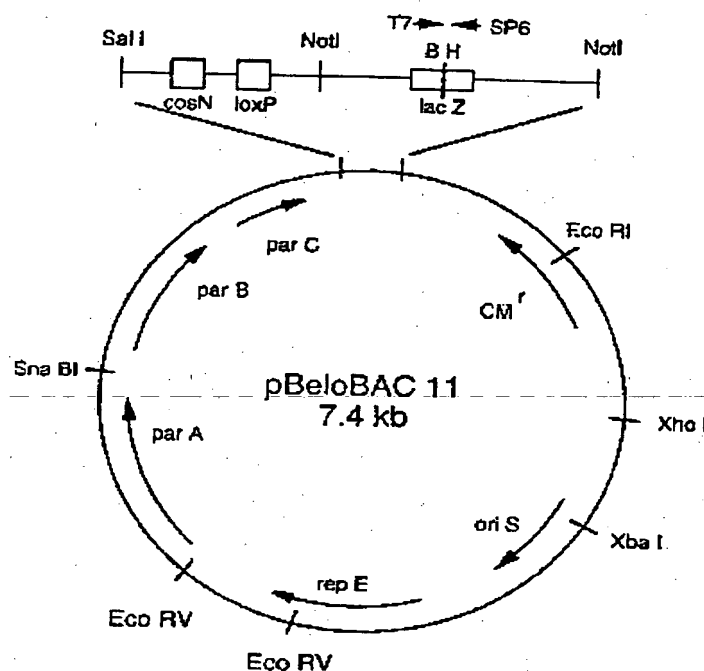
To assemble the gene cluster from cosmids pKOS35-70.1A2, and pKOS35-79.85, small regions of homology from these cosmids have to be introduced into *Myxococcus*  
10 *xanthus* to provide recombination sites for larger pieces of the gene cluster. As shown in Figure 4, plasmids pKOS35-154 and pKOS90-22 are created to introduce these recombination sites. The strategy for assembling the epothilone gene cluster in the *M. xanthus* chromosome is shown in Figure 5. Initially, a neutral site in the bacterial chromosome is chosen that does not disrupt any genes or transcriptional units. One such  
15 region is downstream of the devS gene, which has been shown not to affect the growth or development of *M. xanthus*. The first plasmid, pKOS35-154, is linearized with DraI and electroporated into *M. xanthus*. This plasmid contains two regions of the dev locus flanking two fragments of the epothilone gene cluster. Inserted in between the epo gene regions are the kanamycin resistance marker and the galK gene. Kanamycin resistance  
20 arises in colonies if the DNA recombines into the dev region by a double recombination using the dev sequence as regions of homology. This strain, K35-159, contains small regions of the epothilone gene cluster that will allow for recombination of pKOS35-79.85. Because the resistance markers on pKOS35-79.85 are the same as that for K35-159, a tetracycline transposon was transposed into the cosmid, and cosmids that contain the  
25 transposon inserted into the kanamycin marker were selected. This cosmid, pKOS90-23, was electroporated into K35-159, and oxytetracycline resistant colonies were selected to create strain K35-174. To remove the unwanted regions from the cosmid and leave only the epothilone genes, cells were plated on CYE plates containing 1% galactose. The presence of the galK gene makes the cells sensitive to 1% galactose. Galactose resistant  
30 colonies of K35-174 represent cells that have lost the galK marker by recombination or by a mutation in the galK gene. If the recombination event occurs, then the galactose resistant strain is sensitive to kanamycin and oxytetracycline. Strains sensitive to both antibiotics are verified by Southern blot analysis. The correct strain is identified and designated K35-



175 and contains the epothilone gene cluster from module 7 through two open reading frames past the *epoL* gene.

To introduce modules 1 through module 7, the above process is repeated once more. The plasmid pKOS90-22 is linearized with *DraI* and electroporated into K35-175 to  
5 create K35-180. This strain is electroporated with the tetracycline resistant version of pKOS35-70.1A2, pKOS90-38, and colonies resistant to oxytetracycline are selected. This creates strain K35-185. Recombinants that now have the whole epothilone gene cluster are selected by resistance to 1% galactose. This results in strain K35-188. This strain contains  
10 all the epothilone genes as well as all potential promoters. This strain is fermented and tested for the production of epothilones A and B.

To clone the whole gene cluster as one fragment, a bacterial artificial chromosome (BAC) library is constructed. First, SMP44 cells are embedded in agarose and lysed according to the BIO-RAD genomic DNA plug kit. DNA plugs are partially digested with  
15 restriction enzyme, such as *Sau3AI* or *HindIII*, and electrophoresed on a FIGE or CHEF gel. DNA fragments are isolated by electroeluting the DNA from the agarose or using gelase to degrade the agarose. The method of choice to isolate the fragments is electroelution, as described in Strong *et al.*, 1997, *Nucleic Acids Res.* 19: 3959-3961, incorporated herein by reference. The DNA is ligated into the BAC (pBeloBACII) cleaved with the appropriate enzyme. A map of pBeloBACII is shown below.



The DNA is electroporated into DH10B cells by the method of Sheng *et al.*, 1995, Nucleic Acids Res. 23: 1990-1996, incorporated herein by reference, to create an *S. cellulorum* genomic library. Colonies are screened using a probe from the NRPS region of the epothilone cluster. Positive clones are picked and DNA is isolated for restriction analysis to confirm the presence of the complete gene cluster. This positive clone is designated pKOS35-178.

To create a strain that can be used to introduce pKOS35-178, a plasmid, pKOS35-164, is constructed that contains regions of homology that are upstream and downstream of the epothilone gene cluster flanked by the dev locus and containing the kanamycin resistance galK cassette, analogous to plasmids pKOS90-22 and pKOS35-154. This plasmid is linearized with DraI and electroporated into *M. xanthus*, in accordance with the method of Kafeshi *et al.*, 1995, Mol. Microbiol. 15: 483-494, to create K35-183. The plasmid pKOS35-178 can be introduced into K35-183 by electroporation or by transduction with bacteriophage P1 and chloramphenicol resistant colonies are selected. Alternatively, a version of pKOS35-178 that contains the origin of conjugative transfer from pRP4 can be constructed for transfer of DNA from *E. coli* to K35-183. This plasmid

is made by first constructing a transposon containing the oriT region from RP4 and the tetracycline resistance maker from pACYC184 and then transposing the transposon *in vitro* or *in vivo* onto pKOS35-178. This plasmid is transformed into S17-1 and conjugated into *M. xanthus*. This strain, K35-190, is grown in the presence of 1% galactose to select for the second recombination event. This strain contains all the epothilone genes as well as all potential promoters. This strain will be fermented and tested for the production of epothilones A and B.

Besides integrating pKOS35-178 into the dev locus, it can also be integrated into a phage attachment site using integration functions from myxophages Mx8 or Mx9. A transposon is constructed that contains the integration genes and att site from either Mx8 or Mx9 along with the tetracycline gene from pACYC184. Alternative versions of this transposon may have only the attachment site. In this version, the integration genes are then supplied in trans by coelectroporation of a plasmid containing the integrase gene or having the integrase protein expressed in the electroporated strain from any constitutive promoter, such as the mgl promoter (see Magrini *et al.*, Jul. 1999, J. Bact. 181(13): 4062-4070, incorporated herein by reference). Once the transposon is constructed, it is transposed onto pKOS35-178 to create pKOS35-191. This plasmid is introduced into *Myxococcus xanthus* as described above. This strain contains all the epothilone genes as well as all potential promoters. This strain is fermented and tested for the production of epothilones A and B.

Once the epothilone genes have been established in a strain of *Myxococcus xanthus*, manipulation of any part of the gene cluster, such as changing promoters or swapping modules, can be performed using the kanamycin resistance and galK cassette.

Cultures of *Myxococcus xanthus* containing the epo genes are grown in a number of media and examined for production of epothilones. If the levels of production of epothilones (in particular B or D) are too low to permit large scale fermentation, the *M. xanthus*-producing clones are subjected to media development and strain improvement, as described below for enhancing production in *Streptomyces*.

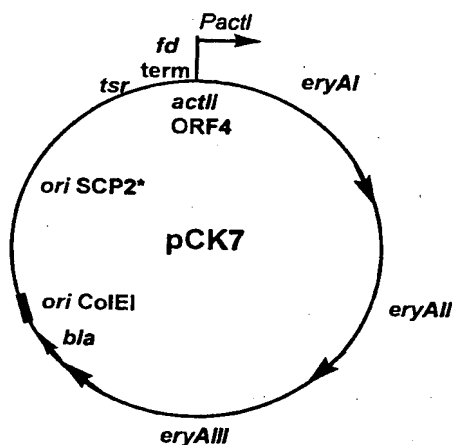
30

#### Example 4

##### Construction of a *Streptomyces* Expression Vector

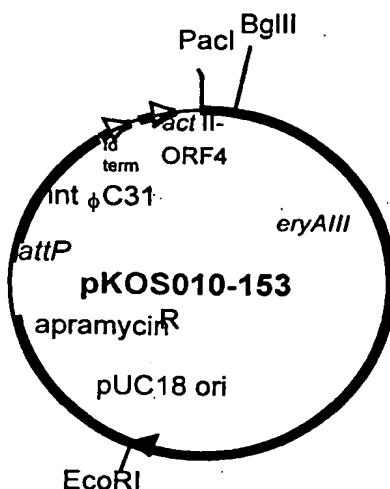
The present invention provides recombinant expression vectors for the heterologous expression of modular polyketide synthase genes in *Streptomyces* hosts.

These vectors include expression vectors that employ the *actI* promoter that is regulated by the gene *actII* ORF4 to allow regulated expression at high levels when growing cells enter stationary phase. Among the vectors available are plasmids pRM1 and pRM5, and derivatives thereof such as pCK7, which are stable, low copy plasmids that carry the marker for thiostrepton resistance in actinomycetes. Such plasmids can accommodate large inserts of cloned DNA and have been used for the expression of the DEBS PKS in *S. coelicolor* and *S. lividans*, the picromycin PKS genes in *S. lividans*, and the oleandomycin PKS genes in *S. lividans*. See U.S. Patent No. 5,712,146. Those of skill in the art recognize that *S. lividans* does not make the tRNA that recognizes the TTA codon for leucine until late-stage growth and that if production of a protein is desired earlier, then appropriate codon modifications can be made.



Plasmid pCK7

Another vector is a derivative of plasmid pSET152 and comprises the *actII* ORF4-PactI expression system but carries the selectable marker for apramycin resistance. These vectors contain the *attP* site and integrase gene of the actinophage phiC31 and do not replicate autonomously in *Streptomyces* hosts but integrate by site specific recombination into the chromosome at the attachment site for phiC31 after introduction into the cell. Derivatives of pCK7 and pSET152 have been used together for the heterologous production of a polyketide, with different PKS genes expressed from each plasmid. See U.S. patent application Serial No. 60/129,731, filed 16 Apr. 1999, incorporated herein by reference.



#### Plasmid pKOS010-153, a pSET152 Derivative

The need to develop expression vectors for the epothilone PKS that function in *Streptomyces* is significant. The epothilone compounds are currently produced in the slow growing, genetically intractable host *Sorangium cellulosum* or are made synthetically. The streptomycetes, bacteria that produce more than 70% of all known antibiotics and important complex polyketides, are excellent hosts for production of epothilones and epothilone derivatives. *S. lividans* and *S. coelicolor* have been developed for the expression of heterologous PKS systems. These organisms can stably maintain cloned heterologous PKS genes, express them at high levels under controlled conditions, and modify the corresponding PKS proteins (e.g. phosphopantetheinylation) so that they are capable of production of the polyketide they encode. Furthermore, these hosts contain the necessary pathways to produce the substrates required for polyketide synthesis, e.g. malonyl CoA and methylmalonyl CoA. A wide variety of cloning and expression vectors are available for these hosts, as are methods for the introduction and stable maintenance of large segments of foreign DNA. Relative to the slow growing *Sorangium* host, *S. lividans* and *S. coelicolor* grow well on a number of media and have been adapted for high level production of polyketides in fermentors. A number of approaches are available for yield improvements, including rational approaches to increase expression rates, increase precursor supply, etc. Empirical methods to increase the titers of the polyketides, long since proven effective for numerous other polyketides produced in streptomycetes, can also be employed for the epothilone and epothilone derivative producing host cells of the invention.

To produce epothilones by fermentation in a heterologous *Streptomyces* host, the epothilone PKS (including the NRPS module) genes are cloned in two segments in derivatives of pCK7 (loading domain through module 6) and pKOS010-153 (modules 7 through 9). The two plasmids are introduced into *S. lividans* employing selection for  
5 thiostrepton and apramycin resistance. In this arrangement, the pCK7 derivative replicates autonomously whereas the pKOS010-153 derivative is integrated in the chromosome. In both vectors, expression of the epothilone genes is from the actI promoter resident within the plasmid.

To facilitate the cloning, the two epothilone PKS encoding segments (one for the  
10 loading domain through module six and one for modules seven through nine) were cloned as translational fusions with the N-terminal segment of the KS domain of module 5 of the ery PKS. High level expression has been demonstrated from this promoter employing KS5 as the first translated sequence, see Jacobsen *et al.*, 1998, Biochemistry 37: 4928-4934, incorporated herein by reference. A convenient BsaBI site is contained within the DNA  
15 segment encoding the amino acid sequence EPIAV that is highly conserved in many KS domains including the KS-encoding regions of *epoA* and of module 7 in *epoE*.

The expression vector for the loading domain and modules one through six of the epothilone PKS was designated pKOS039-124, and the expression vector for modules seven through nine was designated pKOS039-126. Those of skill in the art will recognize  
20 that other vectors and vector components can be used to make equivalent vectors. Because preferred expression vectors of the invention, described below and derived from pKOS039-124 and pKOS039-126, have been deposited under the terms of the Budapest Treaty, only a summary of the construction of plasmids pKOS039-124 and pKOS039-126 is provided below.

25 The eryKS5 linker coding sequences were cloned as an ~0.4 kb PacI-BglII restriction fragment from plasmid pKOS10-153 into pKOS039-98 to construct plasmid pKOS039-117. The coding sequences for the eryKS5 linker were linked to those for the epothilone loading domain by inserting the ~8.7 kb EcoRI-XbaI restriction fragment from cosmid pKOS35-70.1A2 into EcoRI-XbaI digested plasmid pLItmus28. The ~3.4 kb of  
30 BsaBI-NotI and ~3.7 kb NotI-HindIII restriction fragments from the resulting plasmid were inserted into BsaBI-HindIII digested plasmid pKOS039-117 to construct plasmid pKOS039-120. The ~7 kb PacI-XbaI restriction fragment of plasmid pKOS039-120 was inserted into plasmid pKAO18' to construct plasmid pKOS039-123. The final pKOS039-

124 expression vector was constructed by ligating the ~34 kb XbaI-AvrII restriction fragment of cosmid pKOS35-70.1A2 with the ~21.1 kb AvrII-XbaI restriction fragment of pKOS039-123.

5 The plasmid pKOS039-126 expression vector was constructed as follows. First the coding sequences for module 7 were linked from cosmids pKOS35-70.4 and pKOS35-79.85 by cloning the ~6.9 kb BglII-NotI restriction fragment of pKOS35-70.4 and the ~5.9 kb NotI-HindIII restriction fragment of pKOS35-79.85 into BglII-HindIII digested plasmid pLitmus28 to construct plasmid pKOS039-119. The ~12 kb NdeI-NheI restriction fragment of cosmid pKOS35-79.85 was cloned into NdeI-XbaI digested plasmid  
10 pKOS039-119 to construct plasmid pKOS039-122.

To fuse the eryKS5 linker coding sequences with the coding sequences for module 7, the ~1 kb BsaBI-BglII restriction fragment derived from cosmid pKOS35-70.4 was cloned into BsaBI-BclI digested plasmid pKOS039-117 to construct plasmid pKOS039-121. The ~21.5 kb AvrII restriction fragment from plasmid pKOS039-122 was cloned into  
15 AvrII-XbaI digested plasmid pKOS039-121 to construct plasmid pKOS039-125. The ~21.8 kb PacI-EcoRI restriction fragment of plasmid pKOS039-125 was ligated with the ~9 kb PacI-EcoRI restriction fragment of plasmid pKOS039-44 to construct pKOS039-126.

Plasmids pKOS039-124 and pKOS126 were introduced into *S. lividans* K4-114  
20 sequentially employing selection for the corresponding drug resistance marker. Because plasmid pKOS039-126 does not replicate autonomously in streptomycetes, the selection is for cells in which the plasmid has integrated in the chromosome by site-specific recombination at the attB site of phiC31. Because the plasmid stably integrates, continued selection for apramycin resistance is not required. Selection can be maintained if desired.  
25 The presence of thiostrepton in the medium is maintained to ensure continued selection for plasmid pKOS039-124. Plasmids pKOS039-124 and pKOS039-126 were transformed into *Streptomyces lividans* K4-114, and transformants containing the plasmids were cultured and tested for production of epothilones. Initial tests did not indicate the presence of an epothilone.

30 To improve production of epothilones from these vectors, the eryKS5 linker sequences were replaced by epothilone PKS gene coding sequences, and the vectors were introduced into *Streptomyces coelicolor* CH999. To amplify by PCR coding sequences from the *epoA* gene coding sequence, two oligonucleotides primers were used:

N39-73, 5'-GCTTAATTAAGGAGGACACATATGCCCCGTCGTGGCGGATCGTCC-3'; and  
N39-74, 5'-GCGGATCCTCGAATCACCGCCAATATC-3'.

The template DNA was derived from cosmid pKOS35-70.8A3. The ~0.8 kb PCR product was digested with restriction enzymes *PacI* and *BamHI* and then ligated with the ~2.4 kb  
5 *BamHI*-*NotI* and the ~6.4 kb *PacI*-*NotI* restriction fragments of plasmid pKOS039-120 to construct plasmid pKOS039-136. To make the expression vector for the *epoA*, *epoB*,  
*epoC*, and *epoD* genes, the ~5 kb *PacI*-*AvrII* restriction fragment of plasmid pKOS039-136 was ligated with the ~50 kb *PacI*-*AvrII* restriction fragment of plasmid pKOS039-124 to construct the expression plasmid pKOS039-124R. Plasmid pKOS039-124R has been  
10 deposited with the ATCC under the terms of the Budapest Treaty and is available under accession number \_\_\_\_\_.

To amplify by PCR sequences from the *epoE* gene coding sequence, two oligonucleotide primers were used:

N39-67A, 5'-GCTTAATTAAGGAGGACACATATGACCGACCGAGAAGGCCAGCTC-CTGGA-3', and  
15 N39-68, 5'-GGACCTAGGCGGGATGCCGGCGTCT-3'.

The template DNA was derived from cosmid pKOS35-70.1A2. The ~0.4 kb amplification product was digested with restriction enzymes *PacI* and *AvrII* and ligated with either the ~29.5 kb *PacI*-*AvrII* restriction fragment of plasmid pKOS039-126 or the ~23.8 kb *PacI*-*AvrII* restriction fragment of plasmid pKOS039-125 to construct plasmid  
20 pKOS039-126R or plasmid pKOS039-125R, respectively. Plasmid pKOS039-126R was deposited with the ATCC under the terms of the Budapest Treaty and is available under accession number \_\_\_\_\_.

The plasmid pair pKOS039-124R and pKOS039-126R (as well as the plasmid pair pKOS039-124 and pKOS039-126) contain the full complement of *epoA*, *epoB*, *epoC*,  
25 *epoD*, *epoE*, *epoF*, *epoK*, and *epoL* genes. The latter two genes are present on plasmid pKOS039-126R (as well as plasmid pKOS039-126); however, to ensure that these genes were expressed at high levels, another expression vector of the invention, plasmid pKOS039-141 (Figure 8), was constructed in which the *epoK* and *epoL* genes were placed under the control of the *ermE\** promoter.

30 The *epoK* gene sequences were amplified by PCR using the oligonucleotide primers:

N39-69, 5'-AGGCATGCATATGACCCAGGAGCAAGCGAATCAGAGTG-3'; and  
N39-70, 5'-CCAAGCTTTATCCAGCTTTGGAGGGCTTCAAG-3'.



The *epoL* gene sequences were amplified by PCR using the oligonucleotide primers:

N39-71A, 5'-GTAAGCTTAGGAGGACACATATGATGCAACTCGCGCGGGTG-3'; and  
N39-72, 5'-GCCTGCAGGCTCAGGCTTGCGCAGAGCGT-3'.

5 The template DNA for the amplifications was derived from cosmid pKOS35-79.85. The PCR products were subcloned into PCR-script for sequence analysis. Then, the *epoK* and *epoL* genes were isolated from the clones as NdeI-HindIII and HindIII-EcoRI restriction fragments, respectively, and ligated with the ~6 kb NdeI-EcoRI restriction fragment of plasmid pKOS039-134B, which contains the *ermE\** promoter, to construct  
10 plasmid pKOS039-140. The ~2.4 kb NheI-PstI restriction fragment of plasmid pKOS039-140 was cloned into XbaI-PstI digested plasmid pSAM-Hyg, a plasmid pSAM2 derivative containing a hygromycin resistance conferring gene, to construct plasmid pKOS039-141.

Another variant of plasmid pKOS039-126R was constructed to provide the *epoE* and *epoF* genes on an expression vector without the *epoK* and *epoL* genes. This plasmid,  
15 pKOS045-12 (Figure 9), was constructed as follows. Plasmid pXH106 (described in J. Bact., 1991, 173: 5573-5577, incorporated herein by reference) was digested with restriction enzymes StuI and BamHI, and the ~2.8 kb restriction fragment containing the *xylE* and hygromycin resistance conferring genes was isolated and cloned into EcoRV-BglII digested plasmid pLitmus28. The ~2.8 kb NcoI-AvrII restriction fragment of the  
20 resulting plasmid was ligated to the ~18 kb PacI-BspHI restriction fragment of plasmid pKOS039-125R and the ~9 kb SpeI-PacI restriction fragment of plasmid pKOS039-42 to construct plasmid pKOS045-12.

To construct an expression vector that comprised only the *epoL* gene, plasmid pKOS039-141 was partially digested with restriction enzyme NdeI, the ~9 kb NdeI  
25 restriction fragment was isolated, and the fragment then circularized by ligation to yield plasmid pKOS039-150.

The various expression vectors described above were then transformed into *Streptomyces coelicolor* CH999 and *S. lividans* K4-114 in a variety of combinations, the transformed host cells fermented on plates and in liquid culture (R5 medium, which is  
30 identical to R2YE medium without agar). Typical fermentation conditions follow. First, a seed culture of about 5 mL containing 50 µg/L thiostrepton was inoculated and grown at 30°C for two days. Then, about 1 to 2 mL of the seed culture was used to inoculate a production culture of about 50 mL containing 50 µg/L thiostrepton and 1 mM cysteine,

and the production culture was grown at 30°C for 5 days. Also, the seed culture was used to prepare plates of cells (the plates contained the same media as the production culture with 10 mM propionate), which were grown at 30°C for nine days.

Certain of the *Streptomyces coelicolor* cultures and culture broths were analyzed for production of epothilones. The liquid cultures were extracted with three times with equal volumes of ethyl acetate, the organic extracts combined and evaporated, and the residue dissolved in acetonitrile for LC/MS analysis. The agar plate media was chopped and extracted twice with equal volumes of acetone, and the acetone extracts were combined and evaporated to an aqueous slurry, which was extracted three times with equal volumes of ethyl acetate. The organic extracts were combined and evaporated, and the residue dissolved in acetonitrile for LC/MS analysis.

Production of epothilones was assessed using LC-mass spectrometry. The output flow from the UV detector of an analytical HPLC was split equally between a Perkin-Elmer/Sciex API100LC mass spectrometer and an Alltech 500 evaporative light scattering detector. Samples were injected onto a 4.6 x 150 mm reversed phase HPLC column (MetaChem 5 m ODS-3 Inertsil) equilibrated in water with a flow rate of 1.0 mL/min. UV detection was set at 250 nm. Sample components were separated using H<sub>2</sub>O for 1 minute, then a linear gradient from 0 to 100% acetonitrile over 10 minutes. Under these conditions, epothilone A elutes at 10.2 minutes and epothilone B elutes at 10.5 minutes. The identity of these compounds was confirmed by the mass spectra obtained using an atmospheric chemical ionization source with orifice and ring voltages set at 75 V and 300 V, respectively, and a mass resolution of 0.1 amu. Under these conditions, epothilone A shows [M+H] at 494.4 amu, with observed fragments at 476.4, 318.3, and 306.4 amu. Epothilone B shows [M+H] at 508.4 amu, with observed fragments at 490.4, 320.3, and 302.4 amu.

Transformants containing the vector pairs pKOS039-124R and pKOS039-126R or pKOS039-124 and pKOS039-126R produced detectable amounts of epothilones A and B. Transformants containing these plasmid pairs and the additional plasmid pKOS039-141 produced similar amounts of epothilones A and B, indicating that the additional copies of the *epoK* and *epoL* genes were not required for production under the test conditions employed. Thus, these transformants produced epothilones A and B when recombinant *epoA*, *epoB*, *epoC*, *epoD*, *epoE*, *epoF*, *epoK*, and *epoL* genes were present. In some

cultures, it was observed that the absence of propionate increased the proportion of epothilone B to epothilone A.

Transformants containing the plasmid pair pKOS039-124R and pKOS045-12 produced epothilones C and D, as did transformants containing this plasmid pair and the additional plasmid pKOS039-150. These results showed that the *epoL* gene was not required under the test conditions employed to form the C-12-C-13 double bond. These results indicate that either the epothilone PKS gene alone is able to form the double bond or that *Streptomyces coelicolor* expresses a gene product able to convert epothilones G and H to epothilones C and D. Thus, these transformants produced epothilones C and D when recombinant *epoA*, *epoB*, *epoC*, *epoD*, *epoE*, and *epoF* genes were present.

The heterologous expression of the epothilone PKS described herein is believed to represent the recombinant expression of the largest proteins and active enzyme complex that have ever been expressed in a recombinant host cell. The epothilone producing *Streptomyces coelicolor* transformants exhibited growth characteristics indicating that either the epothilone PKS genes, or their products, or the epothilones inhibited cell growth or were somewhat toxic to the cells. Any such inhibition or toxicity could be due to accumulation of the epothilones in the cell, and it is believed that the native *Sorangium* producer cells may contain transporter proteins that in effect pump epothilones out of the cell. Such transporter genes are believed to be included among the ORFs located downstream of the *epoK* gene and described above. Thus, the present invention provides *Streptomyces* and other host cells that include recombinant genes that encode the products of one or more, including all, of the ORFs in this region.

For example, each ORF can be cloned behind the *ermE*\* promoter, see Stassi *et al.*, 1998, Appl. Microbiol. Biotechnol. 49: 725-731, incorporated herein by reference, in a pSAM2-based plasmid that can integrate into the chromosome of *Streptomyces coelicolor* and *S. lividans* at a site distinct from *attB* of phage phiC31, see Smokvina *et al.*, 1990, Gene 94: 53-59, incorporated herein by reference. A pSAM2-based vector carrying the gene for hygromycin resistance is modified to carry the *ermE*\* promoter along with additional cloning sites. Each ORF downstream is PCR cloned into the vector which is then introduced into the host cell (also containing pKOS039-124R and pKOS039-126R or other expression vectors of the invention) employing hygromycin selection. Clones carrying each individual gene downstream from *epoK* are analyzed for increased production of epothilones.

Additional fermentation and strain improvement efforts can be conducted as illustrated by the following. The levels of expression of the PKS genes in the various constructs can be measured by assaying the levels of the corresponding mRNAs (by quantitative RT PCR) relative to the levels of another heterologous PKS mRNA (e.g. picromycin) produced from genes cloned in similar expression vectors in the same host. If one of the epothilone transcripts is underproduced, experiments to enhance its production by cloning the corresponding DNA segment in a different expression vector are conducted. for example, multiple copies of any one or more of the epothilone PKS genes can be introduced into a cell if one or more gene products are rate limiting for biosynthesis. If the basis for low level production is not related to low level PKS gene expression (at the RNA level), an empirical mutagenesis and screening approach that is the backbone of yield improvement of every commercially important fermentation product is undertaken. Spores are subjected to UV, X-ray or chemical mutagens, and individual survivors are plated and picked and tested for the level of compound produced in small scale fermentations. Although this process can be automated, one can examine several thousand isolates for quantifiable epothilone production using the susceptible fungus *Mucor hiemalis* as a test organism.

Another method to increase the yield of epothilones produced is to change the KS<sup>Y</sup> domain of the loading domain of the epothilone PKS to a KS<sup>Q</sup> domain. Such altered loading domains can be constructed in any of a variety of ways, but one illustrative method follows. Plasmid pKOS39-124R of the invention can be conveniently used as a starting material. To amplify DNA fragments useful in the construction, four oligonucleotide primers are employed:

N39-83: 5'-CCGGTATCCACCGCGACACACGGC-3',  
N39-84: 5'-GCCAGTCGTCCTCGCTCGTGGCCGTTC-3',  
and N39-73 and N39-74, which have been described above. The PCR fragment generated with N37-73 and N39-83 and the PCR fragment generated with N39-74 and N39-84 are treated with restriction enzymes PacI and BamHI, respectively, and ligated with the ~3.1 kb PacI-BamHI fragment of plasmid pKOS39-120 to construct plasmid pKOS039-148.

The ~0.8 kb PacI-BamHI restriction fragment of plasmid pKOS039-148 (comprising the two PCR amplification products) is ligated with the ~2.4 kb BamHI-NotI restriction fragment and the ~6.4 kb PacI-NotI restriction fragment of plasmid pKOS39-120 to construct pKOS39-136Q. The ~5 kb PacI-AvrII restriction fragment of plasmid pKOS039-

136Q is ligated to the ~50 kb *PacI*-*AvrII* restriction fragment of plasmid pKOS039-124 to construct plasmid pKOS39-124Q. Plasmids pKOS039-124Q and pKOS039-126R are then transformed into *Streptomyces coelicolor* CH999 for epothilone production.

5 The *epoA* through *epoF*, optionally with *epoK* or with *epoK* plus *epoL*, genes cloned and expressed are sufficient for the synthesis of epothilone compounds, and the distribution of the C-12 H to C-12 methyl congeners appears to be similar to that seen in the natural host (A:B::2:1). This ratio reflects that the AT domain of module 4 more closely resembles that of the malonyl rather than methylmalonyl specifying AT consensus domains. Thus, epothilones D and B are produced at lower quantities than their C-12  
10 unmethylated counterparts C and A. The invention provides PKS genes that produce epothilone D and/or B exclusively. Specifically, methylmalonyl CoA specifying AT domains from a number of sources (e.g. the narbonolide PKS, the rapamycin PKS, and others listed above) can be used to replace the naturally occurring AT domain in module 4. The exchange is performed by direct cloning of the incoming DNA into the appropriate  
15 site in the epothilone PKS encoding DNA segment or by gene replacement through homologous recombination.

For gene replacement through homologous recombination, the donor sequence to be exchanged is placed in a delivery vector between segments of at least 1 kb in length that flank the AT domain of *epo* module 4 encoding DNA. Crossovers in the homologous  
20 regions result in the exchange of the *epo* AT4 domain with that on the delivery vector. Because pKOS039-124 and pKOS039-124R contain AT4 coding sequences, they can be used as the host DNA for replacement. The adjacent DNA segments are cloned in one of a number of *E. coli* plasmids that are temperature sensitive for replication. The heterologous AT domains can be cloned in these plasmids in the correct orientation between the  
25 homologous regions as cassettes enabling the ability to perform several AT exchanges simultaneously. The reconstructed plasmid (pKOS039-124\* or pKOS039-124R\*) is tested for ability to direct the synthesis of epothilone B and/or by introducing it along with pKOS039-126 or pKOS039-126R in *Streptomyces coelicolor* and/or *S. lividans*.

Because the titers of the polyketide can vary from strain to strain carrying the  
30 different gene replacements, the invention provides a number of heterologous methylmalonyl CoA specifying AT domains to ensure that production of epothilone D at titers equivalent to that of the C and D mixture produced in the *Streptomyces coelicolor* host described above. In addition, larger segments of the donor genes can be used for the

replacements, including, in addition to the AT domain, adjacent upstream and downstream sequences that correspond to an entire module. If an entire module is used for the replacement, the KS, methylmalonyl AT, DH, KR, ACP – encoding DNA segment can be obtained from for example and without limitation the DNA encoding the tenth module of the rapamycin PKS, or the first or fifth modules of the FK-520 PKS.

### Example 5

#### Heterologous Expression of EpoK and Conversion of Epothilone D to Epothilone B

This Example describes the construction of *E. coli* expression vectors for *epoK*.  
10 The *epoK* gene product was expressed in *E. coli* as a fusion protein with a polyhistidine tag (his tag). The fusion protein was purified and used to convert epothilone D to epothilone B.

Plasmids were constructed to encode fusion proteins composed of six histidine residues fused to either the amino or carboxy terminus of EpoK. The following oligos  
15 were used to construct the plasmids:

55-101.a-1:

5'-AAAAACATATGCACCACCACCACCACATGACACAGGAGCAAGCGAAT-CAGAGTGAG-3',

55-101.b:

5'-AAAAAGGATCCTTAATCCAGCTTTGGAGGGCTT-3',

20 55-101.c:

5'-AAAAACATATGACACAGGAGCAAGCGAAT-3', and

55-101.d:

5'-AAAAAGGATCCTTAGTGGTGGTGGTGGTGGTCCAGCTTTGGAGGGCTTC-AAGATGAC-3'.

The plasmid encoding the amino terminal his tag fusion protein, pKOS55-121, was  
25 constructed using primers 55-101.a-1 and 55-101.b, and the one encoding the carboxy terminal his tag, pKOS55-129, was constructed using primers 55-101.c and 55-101.d in PCR reactions containing pKOS35-83.5 as the template DNA. Plasmid pKOS35-83.5 contains the ~5 kb NotI fragment comprising the *epoK* gene ligated into pBluescriptSKII+ (Stratagene). The PCR products were cleaved with restriction enzymes BamHI and NdeI  
30 and ligated into the BamHI and NdeI sites of pET22b (Invitrogen). Both plasmids were sequenced to verify that no mutations were introduced during the PCR amplification. Protein gels were run as known in the art.

Purification of EpoK was performed as follows. Plasmids pKOS55-121 and pKOS55-129 were transformed into BL21(DE3) containing the groELS expressing

plasmid pREP4-groELS (Caspers *et al.*, 1994, Cellular and Molecular Biology 40(5): 635-644). The strains were inoculated into 250 mL of M9 medium supplemented with 2 mM MgSO<sub>4</sub>, 1% glucose, 20 mg thiamin, 5 mg FeCl<sub>2</sub>, 4 mg CaCl<sub>2</sub> and 50 mg levulinic acid. The cultures were grown to an OD<sub>600</sub> between 0.4 and 0.6, at which point IPTG was added to 1 mM, and the cultures were allowed to grow for an additional two hours. The cells were harvested and frozen at -80°C. The frozen cells were resuspended in 10 ml of buffer 1 (5 mM imidazole, 500 mM NaCl, and 45 mM Tris pH 7.6) and were lysed by sonicating three times for 15 seconds each on setting 8. The cellular debris was pelleted by spinning in an SS-34 rotor at 16,000 rpm for 30 minutes. The supernatant was removed and spun again at 16,000 rpm for 30 minutes. The supernatant was loaded onto a 5 mL nickel column (Novagen), after which the column was washed with 50 mL of buffer 1 (Novagen). EpoK was eluted with a gradient from 5 mM to 1M imidazole. Fractions containing EpoK were pooled and dialyzed twice against 1 L of dialysis buffer (45 mM Tris pH7.6, 0.2 mM DTT, 0.1 mM EDTA, and 20% glycerol). Aliquots were frozen in liquid nitrogen and stored at -80°C. The protein preparations were greater than 90% pure.

The EpoK assay was performed as follows (See Betlach *et al.*, *Biochem* (1998) 37:14937, incorporated herein by reference). Briefly, reactions consisted of 50 mM Tris (pH7.5), 21 µM spinach ferredoxin, 0.132 units of spinach ferredoxin: NADP<sup>+</sup> oxidoreductase, 0.8 units of glucose-6-phosphate dehydrogenase, 1.4 mM NADP, and 7.1 mM glucose-6-phosphate, 100 µM or 200 µM epothilone D (a generous gift of S. Danishefsky), and 1.7 µM amino terminal his tagged EpoK or 1.6 µM carboxy terminal his tagged EpoK in a 100 µL volume. The reactions were incubated at 30°C for 67 minutes and stopped by heating at 90°C for 2 minutes. The insoluble material was removed by centrifugation, and 50 µL of the supernatant were analyzed by LC/MS. HPLC conditions: Metachem 5 µ ODS-3 Inertsil (4.6 X 150 mm); 80% H<sub>2</sub>O for 1 min, then to 100% MeCN over 10 min at 1 mL/min, with UV ( $\lambda_{\text{max}}$ =250 nm), ELSD, and MS detection. Under these conditions, epothilone D eluted at 11.6 min and epothilone B at 9.3 min. the LC/MS spectra were obtained using an atmosphere pressure chemical ionization source with orifice and ring voltages set at 20 V and 250 V, respectively, at a mass resolution of 1 amu. Under these conditions, epothilone E shows an [M+H] at  $m/z$  493, with observed fragments at 405 and 304. Epothilone B shows an [M+H] at  $m/z$  509, with observed fragments at 491 and 320.

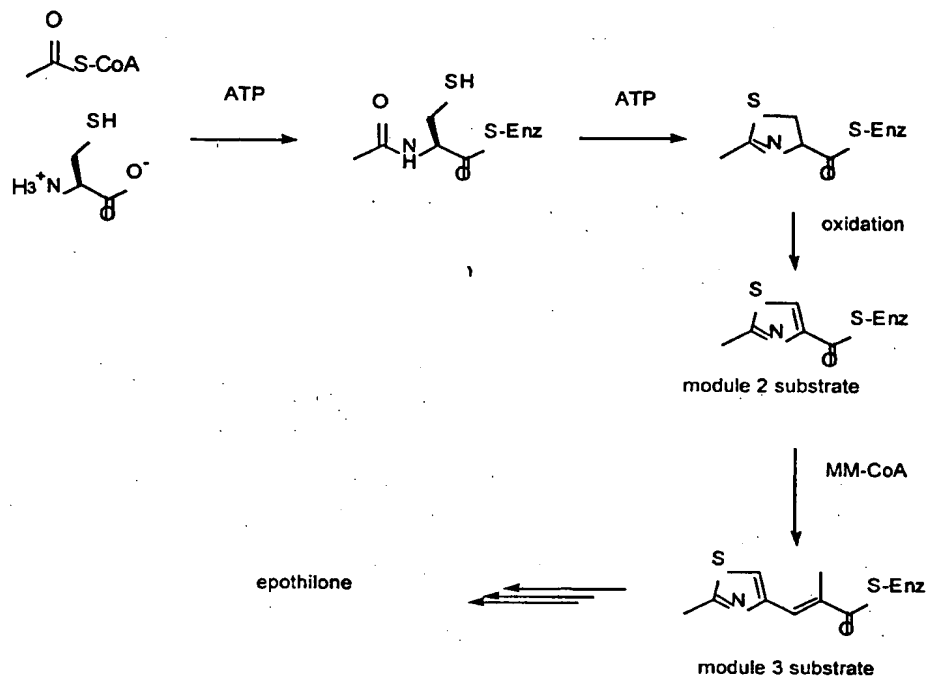
The reactions containing EpoK and epothilone D contained a compound absent in the control that displayed the same retention time, molecular weight, and mass fragmentation pattern as pure epothilone B. With an epothilone D concentration of 100  $\mu$ M, the amino and the carboxy terminal his tagged EpoK was able to convert 82% and 58% to epothilone B, respectively. In the presence of 200  $\mu$ M, conversion was 44% and 21%, respectively. These results demonstrate that EpoK can convert epothilone D to epothilone B.

#### Example 6

##### Modified Epothilones from Chemobiosynthesis

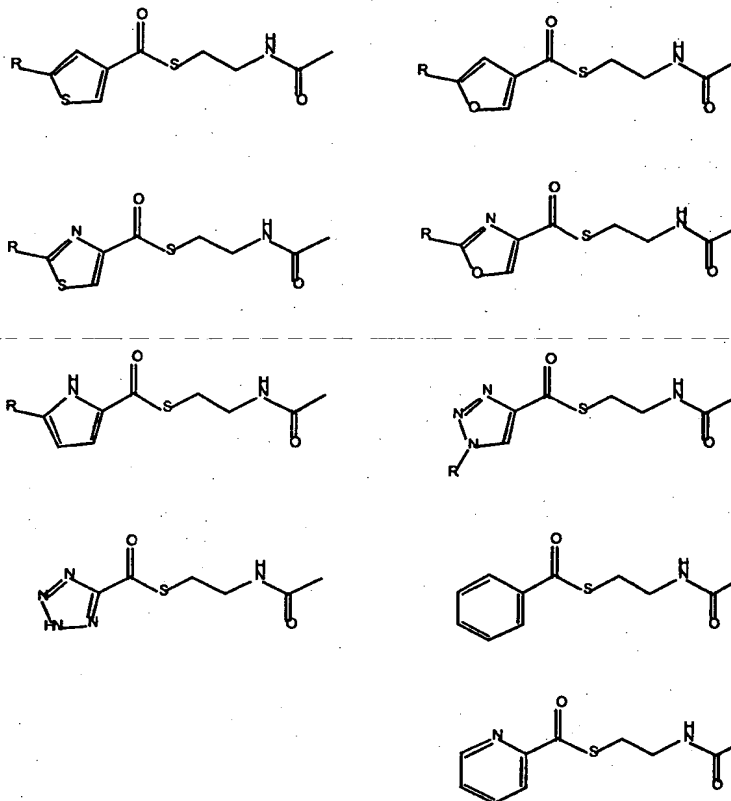
This Example describes a series of thioesters provided by the invention for production of epothilone derivatives via chemobiosynthesis. The DNA sequence of the biosynthetic gene cluster for epothilone from *Sorangium cellulosum* indicates that priming of the PKS involves a mixture of polyketide and amino acid components. Priming involves loading of the PKS-like portion of the loading domain with malonyl CoA followed by decarboxylation and loading of the module one NRPS with cysteine, then condensation to form enzyme-bound N-acetylcysteine. Cyclization to form a thiazoline is followed by oxidation to form enzyme bound 2-methylthiazole-4-carboxylate, the product of the loading domain and NRPS. Subsequent condensation with methylmalonyl CoA by the ketosynthase of module 2 provides the substrate for module, as shown in the following diagram.





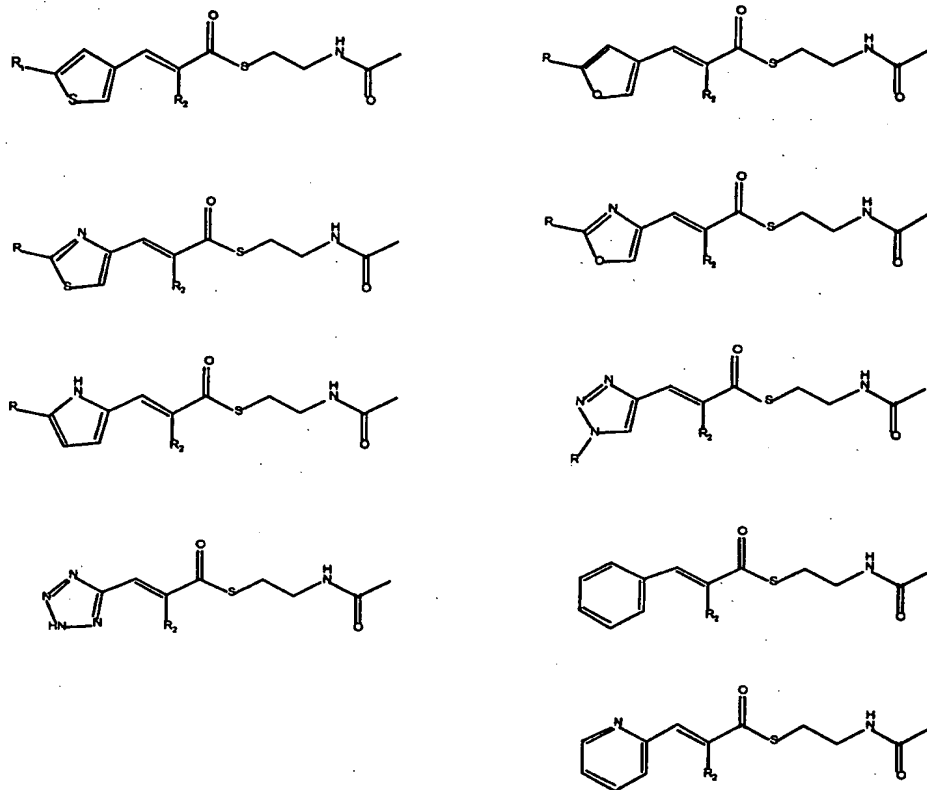
The present invention provides methods and reagents for chemobiosynthesis to produce epothilone derivatives in a manner similar to that described to make 6-dEB and erythromycin analogs in PCT Pat. Pub. Nos. 99/03986 and 97/02358. Two types of feeding substrates are provided: analogs of the NRPS product, and analogs of the module 3 substrate. The module 2 substrates are used with PKS enzymes with a mutated NRPS-like domain, and the module 3 substrates are used with PKS enzymes with a mutated KS domain in module 2.

The following illustrate module 2 substrates (as N-acetyl cysteamine thioesters) for use as substrates for epothilone PKS with modified inactivated NRPS:



5 The module 2 substrates are prepared by activation of the corresponding carboxylic acid and treatment with N-acetylcysteamine. Activation methods include formation of the acid chloride, formation of a mixed anhydride, or reaction with a condensing reagent such as a carbodiimide.

Exemplary module 3 substrates, also as NAc thioesters for use as substrates for epothilone PKS with KS2 knockout are:



These compounds are prepared in a three-step process. First, the appropriate aldehyde is treated with a Wittig reagent or equivalent to form the substituted acrylic ester. The ester is saponified to the acid, which is then activated and treated with N-acetylcysteamine.

Illustrative reaction schemes for making module 2 and module 3 substrates follow. Additional compounds suitable for making starting materials for polyketide synthesis by the epothilone PKS are shown in Figure 2 as carboxylic acids (or aldehydes that can be converted to carboxylic acids) that are converted to the N-acetylcysteamides for supplying to the host cells of the invention.

#### A. Thiophene-3-carboxylate N-acetylcysteamine thioester

A solution of thiophene-3-carboxylic acid (128 mg) in 2 mL of dry tetrahydrofuran under inert atmosphere was treated with triethylamine (0.25 mL) and diphenylphosphoryl azide (0.50 mL). After 1 hour, N-acetylcysteamine (0.25 mL) was added, and the reaction

was allowed to proceed for 12 hours. The mixture was poured into water and extracted three times with equal volumes of ethyl acetate. The organic extracts were combined, washed sequentially with water, 1 N HCl, sat. CuSO<sub>4</sub>, and brine, then dried over MgSO<sub>4</sub>, filtered, and concentrated under vacuum. Chromatography on SiO<sub>2</sub> using ether followed by ethyl acetate provided pure product, which crystallized upon standing.

B. Furan-3-carboxylate N-acetylcysteamine thioester

A solution of furan-3-carboxylic acid (112 mg) in 2 mL of dry tetrahydrofuran under inert atmosphere was treated with triethylamine (0.25 mL) and diphenylphosphoryl azide (0.50 mL). After 1 hour, N-acetylcysteamine (0.25 mL) was added and the reaction was allowed to proceed for 12 hours. The mixture was poured into water and extracted three times with equal volumes of ethyl acetate. The organic extracts were combined, washed sequentially with water, 1 N HCl, sat. CuSO<sub>4</sub>, and brine, then dried over MgSO<sub>4</sub>, filtered, and concentrated under vacuum. Chromatography on SiO<sub>2</sub> using ether followed by ethyl acetate provided pure product, which crystallized upon standing.

C. Pyrrole-2-carboxylate N-acetylcysteamine thioester

A solution of pyrrole-2-carboxylic acid (112 mg) in 2 mL of dry tetrahydrofuran under inert atmosphere was treated with triethylamine (0.25 mL) and diphenylphosphoryl azide (0.50 mL). After 1 hour, N-acetylcysteamine (0.25 mL) was added and the reaction was allowed to proceed for 12 hours. The mixture was poured into water and extracted three times with equal volumes of ethyl acetate. The organic extracts were combined, washed sequentially with water, 1 N HCl, sat. CuSO<sub>4</sub>, and brine, then dried over MgSO<sub>4</sub>, filtered, and concentrated under vacuum. Chromatography on SiO<sub>2</sub> using ether followed by ethyl acetate provided pure product, which crystallized upon standing.

D. 2-Methyl-3-(3-thienyl)acrylate N-acetylcysteamine thioester

(1) Ethyl 2-methyl-3-(3-thienyl)acrylate: A mixture of thiophene-3-carboxaldehyde (1.12 g) and (carbethoxyethylidene)triphenylphosphorane (4.3 g) in dry tetrahydrofuran (20 mL) was heated at reflux for 16 hours. The mixture was cooled to ambient temperature and concentrated to dryness under vacuum. The solid residue was suspended in 1:1 ether/hexane and filtered to remove triphenylphosphine oxide. The

filtrate was filtered through a pad of SiO<sub>2</sub> using 1:1 ether/hexane to provide the product (1.78 g, 91%) as a pale yellow oil.

(2) 2-Methyl-3-(3-thienyl)acrylic acid: The ester from (1) was dissolved in a mixture of methanol (5 mL) and 8 N KOH (5 mL) and heated at reflux for 30 minutes. The mixture was cooled to ambient temperature, diluted with water, and washed twice with ether. The aqueous phase was acidified using 1N HCl then extracted 3 times with equal volumes of ether. The organic extracts were combined, dried with MgSO<sub>4</sub>, filtered, and concentrated to dryness under vacuum. Crystallization from 2:1 hexane/ether provided the product as colorless needles.

(3) 2-Methyl-3-(3-thienyl)acrylate N-acetylcysteamine thioester: A solution of 2-Methyl-3-(3-thienyl)acrylic acid (168 mg) in 2 mL of dry tetrahydrofuran under inert atmosphere was treated with triethylamine (0.56 mL) and diphenylphosphoryl azide (0.45 mL). After 15 minutes, N-acetylcysteamine (0.15 mL) is added and the reaction is allowed to proceed for 4 hours. The mixture is poured into water and extracted three times with equal volumes of ethyl acetate. The organic extracts are combined, washed sequentially with water, 1 N HCl, sat. CuSO<sub>4</sub>, and brine, then dried over MgSO<sub>4</sub>, filtered, and concentrated under vacuum. Chromatography on SiO<sub>2</sub> using ethyl acetate provided pure product, which crystallized upon standing.

The above compounds are supplied to cultures of host cells containing a recombinant epothilone PKS of the invention in which either the NRPS or the KS domain of module 2 as appropriate has been inactivated by mutation to prepare the corresponding epothilone derivative of the invention.

#### Example 7

##### 25 Producing Epothilones and Epothilone Derivatives in *Sorangium cellulosum* SMP44

The present invention provides a variety of recombinant *Sorangium cellulosum* host cells that produce less complex mixtures of epothilones than the naturally occurring epothilone producers as well as host cells that produce epothilone derivatives. This Example illustrates the construction of such strains by describing how to make a strain that produce only epothilones C and D without epothilones A and B. To construct this strain, an inactivating mutation is made in *epoK*. Using plasmid pKOS35-83.5, which contains a NotI fragment harboring the *epoK* gene, the kanamycin and bleomycin resistance markers from Tn5 are ligated into the ScaI site of the *epoK* gene to construct pKOS90-55. The

orientation of the resistance markers is such that transcription initiated at the kanamycin promoter drives expression of genes immediately downstream of *epoK*. In other words, the mutation should be nonpolar. Next, the origin of conjugative transfer, *oriT*, from RP4 is ligated into pKOS90-55 to create pKOS90-63. This plasmid can be introduced into S17-1  
5 and conjugated into SMP44. The transconjugants are selected on phleomycin plates as previously described. Alternatively, electroporation of the plasmid can be achieved using conditions described above for *Myxococcus xanthus*.

Because there are three generalized transducing phages for *Myxococcus xanthus*, one can transfer DNA from *M. xanthus* to SMP44. First, the *epoK* mutation is constructed  
10 in *M. xanthus* by linearizing plasmid pKOS90-55 and electroporating into *M. xanthus*. Kanamycin resistant colonies are selected and have a gene replacement of *epoK*. This strain is infected with Mx9, Mx8, Mx4 ts18 hft hrm phages to make phage lysates. These lysates are then individually infected into SMP44 and phleomycin resistant colonies are selected. Once the strain is constructed, standard fermentation procedures, as described  
15 below, are employed to produce epothilones C and D.

Prepare a fresh plate of *Sorangium* host cells (dispersed) on S42 medium. S42 medium contains tryptone, 0.5 g/L; MgSO<sub>4</sub>, 1.5 g/L; HEPES, 12 g/L; agar, 12 g/L, with deionized water. The pH of S42 medium is set to 7.4 with KOH. To prepare S42 medium, after autoclaving at 121°C for at least 30 minutes, add the following ingredients (per liter):  
20 CaCl<sub>2</sub>, 1 g; K<sub>2</sub>HPO<sub>4</sub>, 0.06 g; Fe Citrate, 0.008 g; Glucose, 3.5 g; Ammonium sulfate, 0.5 g; Spent liquid medium, 35 mL; and 200 micrograms/mL of kanamycin is added to prevent contamination. Incubate the culture at 32°C for 4-7 days, or until orange sporangia appear on the surface.

To prepare a seed culture for inoculating agar plates/bioreactor, the following  
25 protocol is followed. Scrape off a patch of orange *Sorangium* cells from the agar (about 5 mm<sup>2</sup>) and transfer to a 250 ml baffle flask with 38 mm silicone foam closures containing 50 ml of Soymeal Medium containing potato starch, 8 g; defatted soybean meal, 2 g; yeast extract, 2 g; Iron (III) sodium salt EDTA, 0.008 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 1 g; CaCl<sub>2</sub>·2H<sub>2</sub>O, 1 g; glucose, 2 g; HEPES buffer, 11.5 g. Use deionized water, and adjust pH to 7.4 with 10%  
30 KOH. Add 2-3 drops of antifoam B to prevent foaming. Incubate in a coffin shaker for 4-5 days at 30°C and 250 RPM. The culture should appear an orange color. This seed culture can be subcultured repeatedly for scale-up to inoculate in the desired volume of production medium.

The same preparation can be used with Medium 1 containing (per liter)  
CaCl<sub>2</sub>·2H<sub>2</sub>O, 1 g; yeast extract, 2 g; Soytone, 2 g; FeEDTA, 0.008 g; Mg SO<sub>4</sub>·7H<sub>2</sub>O, 1 g;  
HEPES, 11.5 g. Adjust pH to 7.4 with 10% KOH, and autoclave at 121°C for 30 minutes.  
Add 8 ml of 40% glucose after sterilization. Instead of a baffle flask, use a 250 ml coiled  
5 spring flask with a foil cover. Include 2-3 drops of antifoam B, and incubate in a coffin  
shaker for 7 days at 37°C and 250 RPM. Subculture the entire 50 mL into 500 mL of fresh  
medium in a baffled narrow necked Fernbach flask with a 38 mm silicone foam closure.  
Include 0.5 ml of antifoam to the culture. Incubate under the same conditions for 2-3 days.  
Use at least a 10% inoculum for a bioreactor fermentation.

10 To culture on solid media, the following protocol is used. Prepare agar plates  
containing (per liter of CNS medium) KNO<sub>3</sub>, 0.5 g; Na<sub>2</sub>HPO<sub>4</sub>, 0.25 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 1 g;  
FeCl<sub>2</sub>, 0.01 g; HEPES, 2.4 g; Agar, 15 g; and sterile Whatman filter paper. While the agar  
is not completely solidified, place a sterile disk of filter paper on the surface. When the  
plate is dry, add just enough of the seed culture to coat the surface evenly (about 1 mL).  
15 Spread evenly with a sterile loop or an applicator, and place in a 32°C incubator for 7  
days. Harvest plates.

For production in a 5 L bioreactor, the following protocol is used. The  
fermentation can be conducted in a B. Braun Biostat MD-1 5L bioreactor. Prepare 4 L of  
production medium (same as the soymeal medium for the seed culture without HEPES  
20 buffer). Add 2% (volume to volume) XAD-16 absorption resin, unwashed and untreated,  
e.g. add 1 mL of XAD per 50 mL of production medium. Use 2.5 N H<sub>2</sub>SO<sub>4</sub> for the acid  
bottle, 10% KOH for the base bottle, and 50% antifoam B for the antifoam bottle. For the  
sample port, be sure that the tubing that will come into contact with the culture broth has a  
small opening to allow the XAD to pass through into the vial for collecting daily samples.  
25 Stir the mixture completely before autoclaving to evenly distribute the components.  
Calibrate the pH probe and test dissolved oxygen probe to ensure proper functioning. Use  
a small antifoam probe, ~3 inches in length. For the bottles, use tubing that can be sterile  
welded, but use silicone tubing for the sample port. Make sure all fittings are secure and  
the tubings are clamped off, not too tightly, with C-clamps. Do not clamp the tubing to the  
30 exhaust condenser. Attach 0.2 µm filter disks to any open tubing that is in contact with the  
air. Use larger ACRO 50 filter disks for larger tubing, such as the exhaust condenser and  
the air inlet tubing. Prepare a sterile empty bottle for the inoculum. Autoclave at 121°C  
with a sterilization time of 90 minutes. Once the reactor has been taken out of the

autoclave, connect the tubing to the acid, base, and antifoam bottles through their respective pump heads. Release the clamps to these bottles, making sure the tubing has not been welded shut. Attach the temperature probe to the control unit. Allow the reactor to cool, while sparging with air through the air inlet at a low air flow rate.

5 After ensuring the pumps are working and there is no problem with flow rate or clogging, connect the hoses from the water bath to the water jacket and to the exhaust condenser. Make sure the water jacket is nearly full. Set the temperature to 32°C. Connect pH, D.O., and antifoam probes to the main control unit. Test the antifoam probe for proper functioning. Adjust the set point of the culture to 7.4. Set the agitation to 400 RPM.

10 Calibrate the D.O. probe using air and nitrogen gas. Adjust the airflow using the rate at which the fermentation will operate, e.g. 1 LPM (liter per minute). To control the dissolved oxygen level, adjust the parameters under the cascade setting so that agitation will compensate for lower levels of air to maintain a D.O. value of 50%. Set the minimum and maximum agitation to 400 and 1000 RPM respectively, based on the settings of the  
15 control unit. Adjust the settings, if necessary.

Check the seed culture for any contamination before inoculating the fermenter. The *Sorangium cellulosum* cells are rod shaped like a pill, with 2 large distinct circular vacuoles at opposite ends of the cell. Length is approximately 5 times that of the width of the cell. Use a 10% inoculum (minimum) volume, e.g. 400 mL into 4 L of production  
20 medium. Take an initial sample from the vessel and check against the bench pH. If the difference between the fermenter pH and the bench pH is off by  $\geq 0.1$  units, do a 1 point recalibration. Adjust the deadband to 0.1. Take daily 25 mL samples noting fermenter pH, bench pH, temperature, D.O., airflow, agitation, acid, base, and antifoam levels. Adjust pH if necessary. Allow the fermenter to run for seven days before harvesting.

25 Extraction and analysis of compounds is performed substantially as described above in Example 4. In brief, fermentation culture is extracted twice with ethyl acetate, and the ethyl acetate extract is concentrated to dryness and dissolved/suspended in ~500  $\mu$ L of MeCN-H<sub>2</sub>O (1:1). The sample is loaded onto a 0.5 mL Bakerbond ODS SPE cartridge pre-equilibrated with MeCN-H<sub>2</sub>O (1:1). The cartridge is washed with 1 mL of  
30 the same solvent, followed by 2 mL of MeCN. The MeCN eluent is concentrated to dryness, and the residue is dissolved in 200  $\mu$ L of MeCN. Samples (50  $\mu$ L) are analyzed by HPLC/MS on a system comprised of a Beckman System Gold HPLC and PE Sciex API100LC single quadrupole MS-based detector equipped with an atmospheric pressure



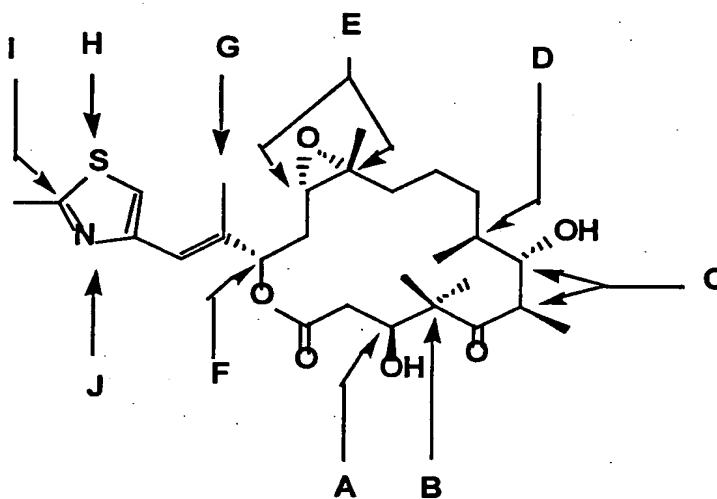
chemical ionization source. Ring and orifice voltages are set to 75V and 300V, respectively, and a dual range mass scan from  $m/z$  290-330 and 450-550 is used. HPLC conditions: Metachem 5 $\mu$  ODS-3 Inertsil (4.6 X 150 mm); 100% H<sub>2</sub>O for 1 min, then to 100% MeCN over 10 min at 1 mL/min. Epothilone A elutes at 0.2 min under these conditions and gives characteristic ions at  $m/z$  494 (M+H), 476 (M+H-H<sub>2</sub>O), 318, and 306.

### Example 8

#### Epothilone Derivatives as Anti-Cancer Agents

The novel epothilone derivatives shown below by Formula (1) set forth above are potent anti-cancer agents and can be used for the treatment of patients with various forms of cancer, including but not limited to breast, ovarian, and lung cancers.

The epothilone structure-activity relationships based on tubulin binding assay are (see Nicolaou *et al.*, 1997, Angew. Chem. Int. Ed. Engl. 36: 2097-2103, incorporated herein by reference) are illustrated by the diagram below.



15

A) (3S) configuration important; B) 4,4-ethano group not tolerated; C) (6R, 7S) configuration crucial; D) (8S) configuration important, 8,8-dimethyl group not tolerated; E) epoxide not essential for tubulin polymerization activity, but may be important for cytotoxicity; epoxide configuration may be important; R group important; both olefin geometries tolerated; F) (15S) configuration important; G) bulkier group reduces activity; H) oxygen substitution tolerated; I) substitution important; J) heterocycle important.

20

Thus, this SAR indicates that modification of the C1-C8 segment of the molecule can have strong effects on activity, whereas the remainder of the molecule is relatively

tolerant to change. Variation of substituent stereochemistry with the C1-C8 segment, or removal of the functionality, can lead to significant loss of activity. Epothilone derivative compounds A-H differ from epothilone by modifications in the less sensitive portion of the molecule and so possess good biological activity and offer better pharmacokinetic characteristics, having improved lipophilic and steric profiles.

These novel derivatives can be prepared by altering the genes involved in the biosynthesis of epothilone optionally followed by chemical modification. The 9-hydroxy-epothilone derivatives prepared by genetic engineering can be used to generate the carbonate derivatives (compound D) by treatment with triphosgene or 1,1'-carbonyldiimidazole in the presence of a base. In a similar manner, the 9,11-dihydroxy-epothilone derivative, upon proper protection of the C-7 hydroxyl group if it is present, yields the carbonate derivatives (compound F). Selective oximation of the 9 oxo-epothilone derivatives with hydroxylamine followed by reduction (Raney nickel in the presence of hydrogen or sodium cyanoborohydride) yield the 9-amino analogs. Reacting these 9-amino derivatives with p-nitrophenyl chloroformate in the presence of base and subsequently reacting with sodium hydride will produce the carbamate derivatives (compound E). Similarly, the carbamate compound G, upon proper protection of the C7 hydroxyl group if it is present, can be prepared from the 9-amino-11 hydroxy-epothilone derivatives.

Illustrative syntheses are provided below.

Part A. Epothilone D-7, 9-cyclic carbonate

To a round bottom flask, a solution of 254 mg epothilone D in 5 mL of methylene chloride is added. It is cooled by an ice bath, and 0.3 mL of triethyl amine is then added. To this solution, 104 mg of triphosgene is added. The ice bath is removed, and the mixture is stirred under nitrogen for 5 hours. The solution is diluted with 20 mL of methylene chloride and washed with dilute sodium bicarbonate solution. The organic solution is dried over magnesium sulfate and filtered. Upon evaporation to dryness, the epothilone D-7, 9 - cyclic carbonate is isolated.

Part B. Epothilone D-7,9-cyclic carbamate

(i) 9-amino-epothilone D

To a rounded bottom flask, a solution of 252 mg 9-oxo-epothilone D in 5 mL of methanol is added. Upon the addition of 0.5 mL 50% hydroxylamine in water and 0.1 mL

acetic acid, the mixture is stirred at room temperature overnight. The solvent is then removed under reduced pressure to yield the 9-oxime-epothilone D. To a solution of this 9 oxime compound in 5 mL of tetrahydrofuran (THF) at ice bath is added 0.25 mL 1M solution of cyanoborohydride in THF. After the mixture is allowed to react for 1 hour, the  
5 ice bath is removed, and the solution is allowed to warm slowly to room temperature. One mL of acetic acid is added, and the solvent is then removed under reduced pressure. The residue is dissolved in 30 mL of methylene chloride and washed with saturated sodium chloride solution. The organic layer is separated and dried over magnesium sulfate and filtered. Upon evaporation of the solvent yields the 9-amino-epothilone D.

10 (ii) Epothilone D-7,9-cyclic carbamate

To a solution of 250 mg of 9-amino-epothilone D in 5 mL of methylene is added 110 mg of 4-nitrophenyl chloroformate followed by the addition of 1 mL of triethylamine. The solution is stirred at room temperature for 16 hours. It is diluted with 25 mL of methylene chloride. The solution is washed with saturated sodium chloride and the organic  
15 layer is separated and dried over magnesium sulfate. After filtration, the solution is evaporated to dryness at reduced pressure. The residue is dissolved in 10 mL of dry THF. Sodium hydride, 40 mg (60% dispersion in mineral oil), is added to the solution in an ice bath. The ice bath is removed, and the mixture is stirred for 16 hours. One-half mL of acetic acid is added, and the solution is evaporated to dryness under reduced pressure. The  
20 residue is re-dissolved in 50 mL methylene chloride and washed with saturated sodium chloride solution. The organic layer is dried over magnesium sulfate and the solution is filtered and the organic solvent is evaporated to dryness under reduced pressure. Upon purification on silica gel column, the epothilone D-7,9-carbamate is isolated.

25 The invention having now been described by way of written description and examples, those of skill in the art will recognize that the invention can be practiced in a variety of embodiments and that the foregoing description and examples are for purposes of illustration and not limitation of the following claims.

Claims

1. An isolated recombinant nucleic acid compound that comprises a nucleotide sequence encoding at least a domain of an epothilone polyketide synthase (PKS) protein and/or encoding a functional region of an epothilone modification enzyme.

2. The nucleic acid of claim 1, wherein said domain is selected from the group consisting of a loading domain, a thioesterase domain, an NRPS, an AT domain, a KS domain, an ACP domain, a KR domain, a DH domain, and an ER domain, a methyl transferase domain and a functional oxidase domain.

3. The nucleic acid of claim 1 or 2 that comprises the coding sequence of an *epoA* gene, and/or  
the coding sequence of an *epoB* gene, and/or  
the coding sequence of an *epoC* gene, and/or  
the coding sequence of an *epoD* gene, and/or  
the coding sequence of an *epoE* gene, and/or  
the coding sequence of an *epoF* gene, and/or  
the coding sequence of an *epoK* gene, and/or  
the coding sequence of an *epoL* gene.

4. The nucleic acid of any of claims 1-3 that further comprises a promoter positioned to transcribe said encoding nucleotide sequence in host cells in which said promoter is operable.

5. The nucleic acid of claim 4, wherein said promoter is a promoter from a *Sorangium* gene, or  
from a *Myxococcus* gene, or  
from a *Streptomyces* gene, or  
from an epothilone PKS gene, or  
from a *pilA* gene, or  
from an actinorhodin PKS gene.

6. The nucleic acid of any of claims 1-5 that is a recombinant DNA expression vector.

7. Host cells which contain the nucleic acid of any of claims 4-6.

8. The cells of claim 7 which are *Sorangium* cells, or *Myxococcus* cells, or *Pseudomonas* cells, or *Streptomyces* cells.

9. A method to produce a polyketide which method comprises culturing the cells of claim 7 or 8 under conditions wherein the encoding nucleotide sequence is expressed to obtain a functional PKS.

10. A recombinant *Sorangium cellulosum* host cell that contains a mutated gene for an epothilone PKS protein or epothilone modification enzyme, wherein said mutated gene was inserted in whole or in part into genomic DNA of said cell by homologous recombination with a recombinant vector comprising all or a part of an epothilone PKS gene or epothilone modification gene.

11. The recombinant host cell of claim 10 that makes epothilone C or D but not A or B due to a mutation inactivating or deleting an *epoK* gene, or

makes epothilone A or C but not B or D due to a mutation in *epoD* altering module 4 AT domain specificity, or

makes epothilone B or D but not A or C due to a mutation in *epoD* altering module 4 AT domain specificity, or

makes epothilone C but not epothilone A, B or D due to a mutation in *epoD* altering module 4 AT domain specificity and a mutation in *epoK*, or

makes epothilone D but not epothilone A, B or C due to a mutation in *epoD* altering module 4 AT domain specificity and a mutation in *epoK*.

12. Recombinant *Streptomyces* or *Myxococcus* host cells that express an epothilone PKS gene or an epothilone modification enzyme gene, optionally comprising one or more of said epothilone PKS or modification enzyme genes integrated into their chromosomal DNA and/or one or more of said epothilone PKS or modification enzyme  
5 genes on an extrachromosomal expression vector.

13. The host cells of claim 12 or 13 that are *S. coelicolor* CH999.

14. A method to produce an epothilone or epothilone derivative which  
10 comprises culturing the cells of claims 12 or 13.

15. A modified functional epothilone PKS wherein said modification comprises at least one of:

15 replacement of at least one AT domain with an AT domain of different specificity;  
inactivation of the NRPS-like module 1 or of the KS2 catalytic domain;  
inactivation of at least one activity in at least one  $\beta$ -carbonyl modification domain;  
addition of at least one of KR, DH and ER activity in at least one  $\beta$ -carbonyl  
modification domain; and  
20 replacement of the NRPS module 1 with an NRPS of different specificity.

16. The modified PKS of claim 15 contained in a cell or contained in a cell-free system, wherein said cell or system contains additional enzymes for modification of the product of said epothilone PKS.

25 17. The modified PKS of claim 16 wherein said modifying enzymes comprise at least one of a methyltransferase, an oxidase or a glycosylation enzyme.

18. A method to prepare an epothilone derivative which method comprises providing substrates including extender units to the modified PKS of any of claims 15-17.  
30

19. A modified functional epothilone PKS wherein said modification comprises inactivation of the NRPS of module 1 or the KS2 of module 2 thereof.

20. A method to make an epothilone derivative which method comprises contacting the modified PKS of claim 19 with a module 2 substrate or a module 3 substrate and extender units.

5        21. Recombinant host cells which comprise the modified PKS of any of claims 15-17 or 19.

22. The cells of claim 21 that produce an epothilone derivative selected from the group consisting of 16-desmethyl epothilones, 14-methyl epothilones, 11-hydroxyl  
10 epothilones, 10-methyl epothilones, 8,9-anhydro epothilones, 9-hydroxyl epothilones, 9-keto epothilones, 8-desmethyl epothilones, and 6-desmethyl epothilones.

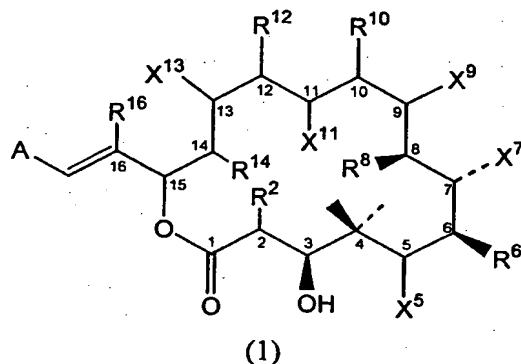
23. A compound selected from the group consisting of 16-desmethyl epothilones, 14-methyl epothilones, 11-hydroxyl epothilones, 10-methyl epothilones, 8,9-  
15 anhydro epothilones, 9-hydroxyl epothilones, 9-keto epothilones, 8-desmethyl epothilones, and 6-desmethyl epothilones.

24. A recombinant PKS enzyme that comprises one or more domains, modules, or proteins of a non-epothilone PKS and one or more domains, modules, or proteins of an  
20 epothilone PKS, and/or

contains a loading domain that comprises a KS<sup>Q</sup> domain.

25. The PKS enzyme of claim 24, wherein  
said PKS comprises a DEBS loading domain and 5 modules of DEBS and an  
NRPS of the epothilone PKS,  
25 wherein said PKS comprises all of a non-epothilone PKS with an MT domain of  
the epothilone PKS

26. A compound of the formula:



including the glycosylated forms thereof and stereoisomeric forms where the

5 stereochemistry is not shown,

wherein A is a substituted or unsubstituted straight, branched chain or cyclic alkyl, alkenyl or alkynyl residue optionally containing 1-3 heteroatoms selected from O, S and N; or wherein A comprises a substituted or unsubstituted aromatic residue;

$R^2$  represents H, H, or H, lower alkyl, or lower alkyl, lower alkyl;

10  $X^5$  represents =O or a derivative thereof, or H, OH or H,  $NR_2$  wherein R is H, alkyl or acyl, or H,  $OCOR_2$ , H,  $OCONR_2$  wherein R is H or alkyl, or is H, H;

$R^6$  represents H or lower alkyl, and the remaining substituent on the corresponding carbon is H;

15  $X^7$  represents OR, or  $NR_2$ , wherein R is H, alkyl or acyl or is  $OCOR$ , or  $OCONR_2$  wherein R is H or alkyl or  $X^7$  taken together with  $X^9$  forms a carbonate or carbamate cycle, and wherein the remaining substituent on the corresponding carbon is H;

$R^8$  represents H or lower alkyl and the remaining substituent on the carbon is H;

20  $X^9$  represents =O or a derivative thereof, or H, OR or H,  $NR_2$  wherein R is H, alkyl or acyl, or is H,  $OCOR$  or H,  $OCONR_2$ , wherein R is H or alkyl, or represents H, H or wherein  $X^9$  together with  $X^7$  or with  $X^{11}$  can form a cyclic carbonate or carbamate;

$R^{10}$  is H, H or H, lower alkyl, or lower alkyl, lower alkyl;

$X^{11}$  is =O or a derivative thereof, or H, OR, or H,  $NR_2$  wherein R is H, alkyl or acyl or H,  $OCOR$  or H,  $OCONR_2$  wherein R is H or alkyl, or is H, H or wherein  $X^{11}$  in combination with  $X^9$  may form a cyclic carbonate or carbamate;

25  $R^{12}$  is H, H, or H, lower alkyl, or lower alkyl, lower alkyl;

$X^{13}$  is =O or a derivative thereof, or H, OR or H,  $NR_2$  wherein R is H, alkyl or acyl or is H,  $OCOR$  or H,  $OCONR_2$  wherein R is H or alkyl;

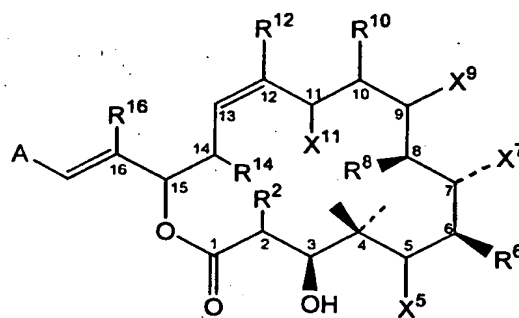


$R^{14}$  is H, H, or H, lower alkyl, or lower alkyl, lower alkyl;

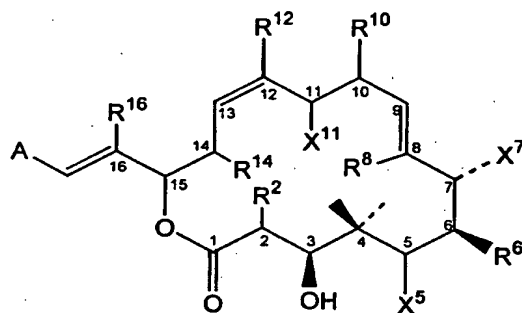
$R^{16}$  is H or lower alkyl; and

wherein optionally H or another substituent may be removed from positions 12 and 13 and/or 8 and 9 to form a double bond, wherein said double bond may optionally be  
 5 converted to an epoxide.

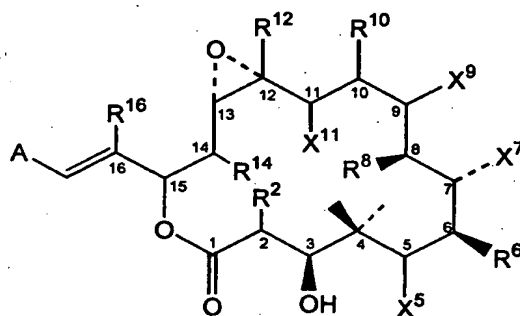
27. A compound of the formula



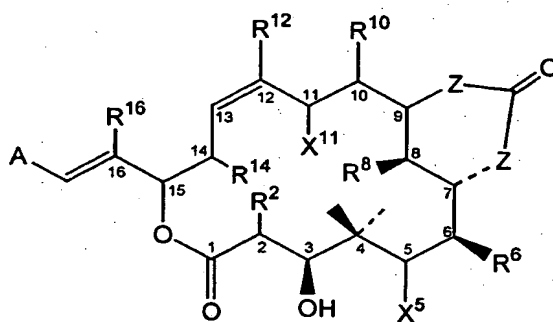
1(a),



1(b),

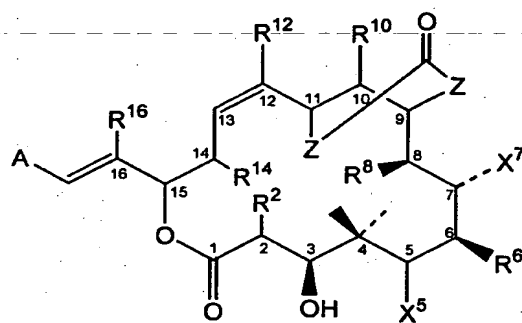


1(c),



1(d),

and



1(e)

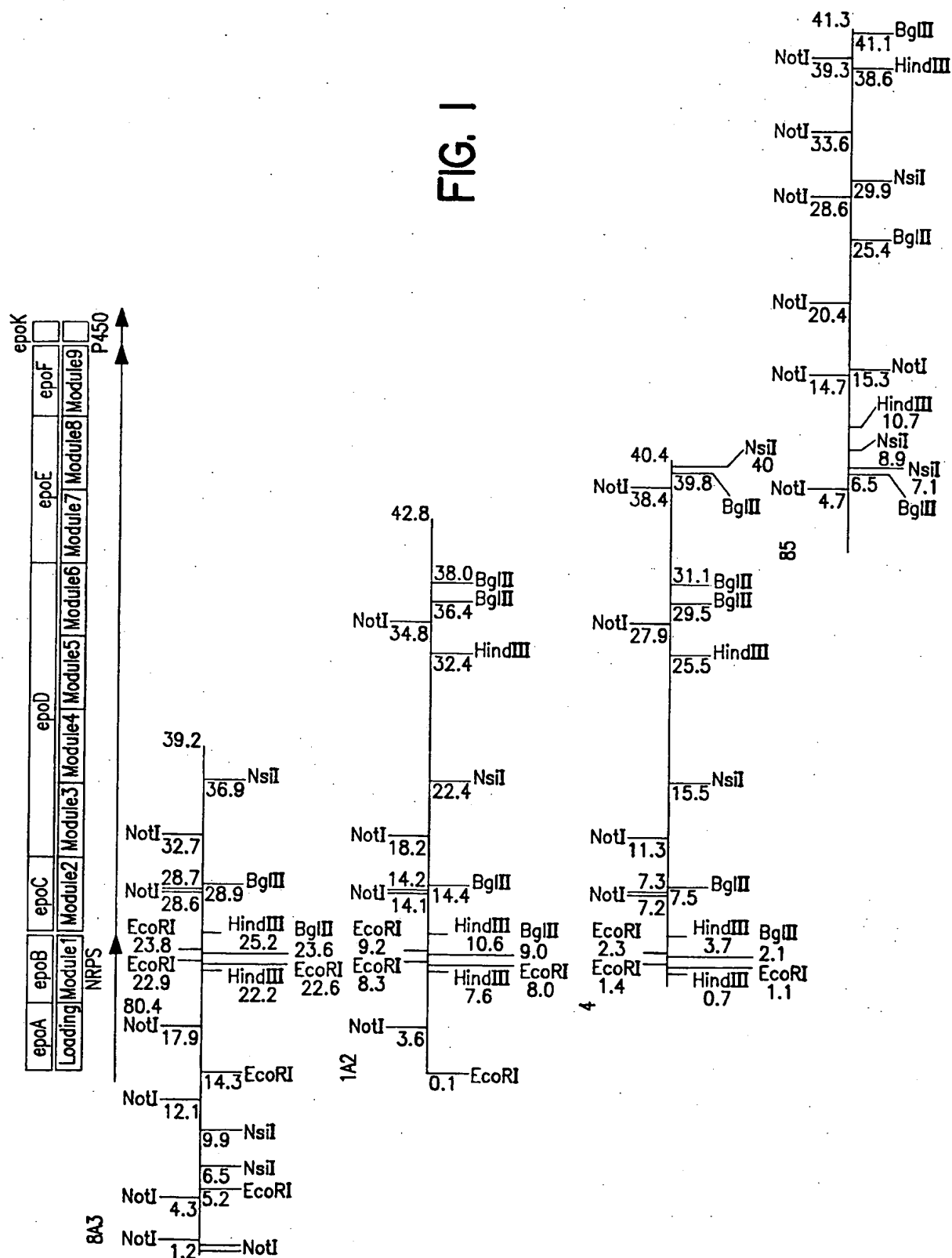
5

wherein both Z are O or one Z is N and the other Z is O and the remaining substituents are defined as in claim 26.

28. A recombinant vector selected from the group consisting of pKOS35-  
 10 70.8A3, pKOS35-70.1A2, pKOS35-70.4, pKOS35-79.85, pKOS039-124R, and  
 pKOS039-126R.

1 / 8

FIG. 1



2 / 8

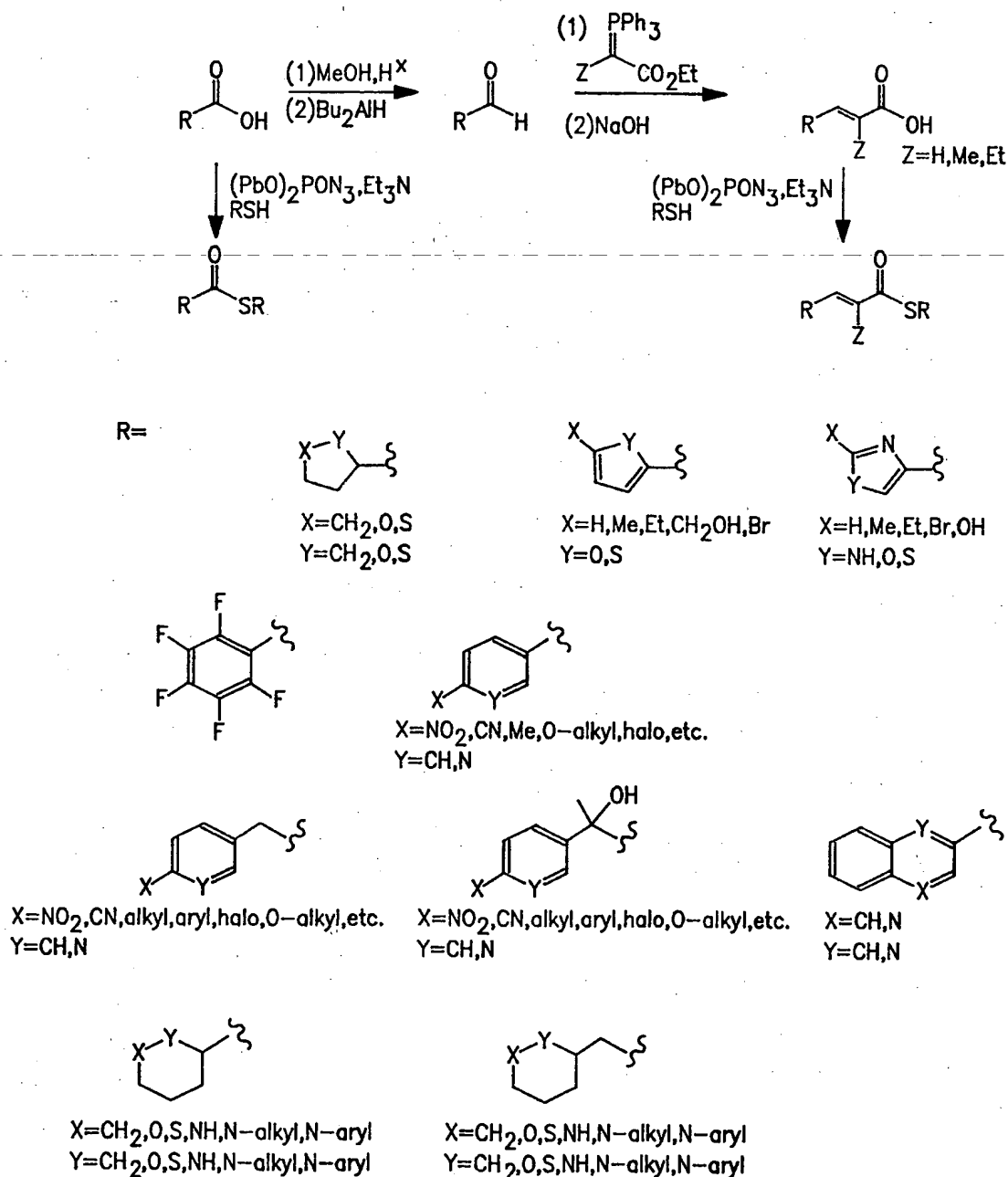


FIG. 2

Alternative Primers for Biosynthetic Epothilone Analogs

3 / 8

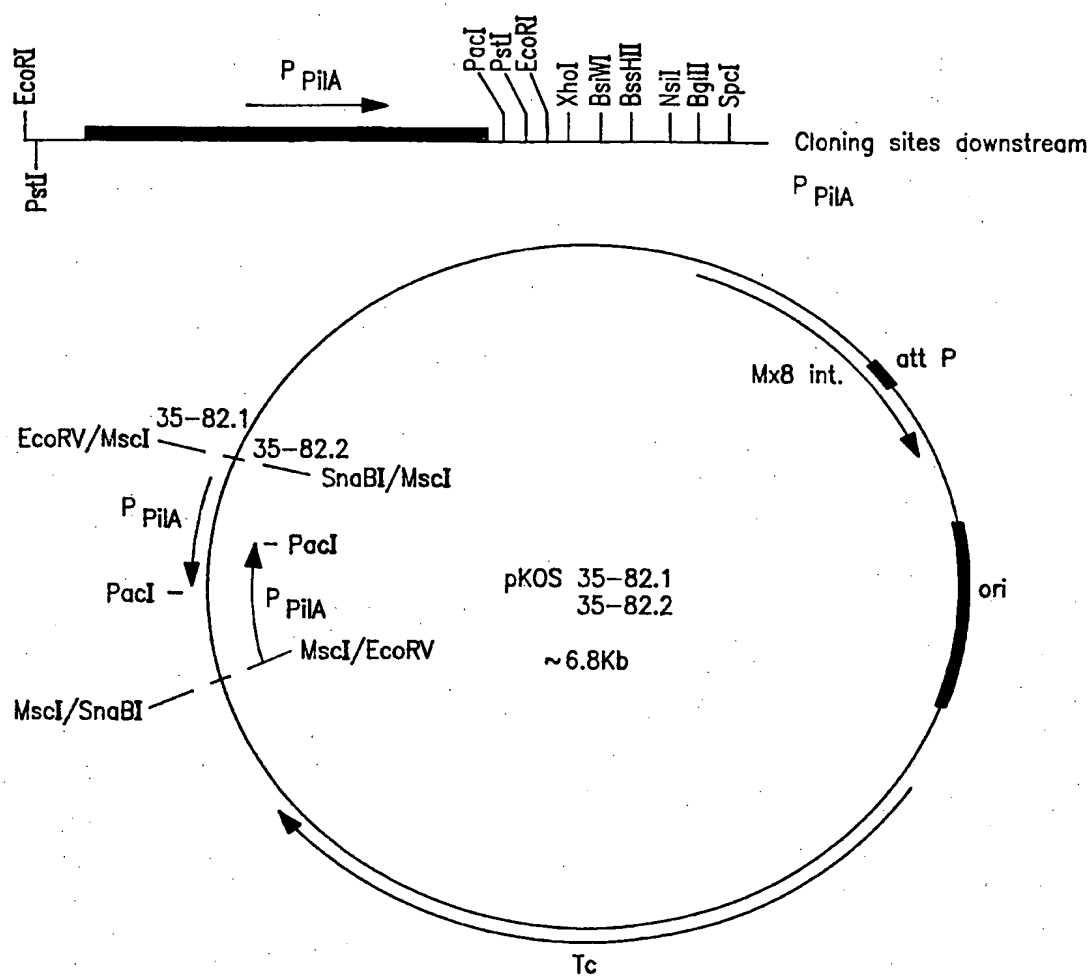


FIG. 3

4/8

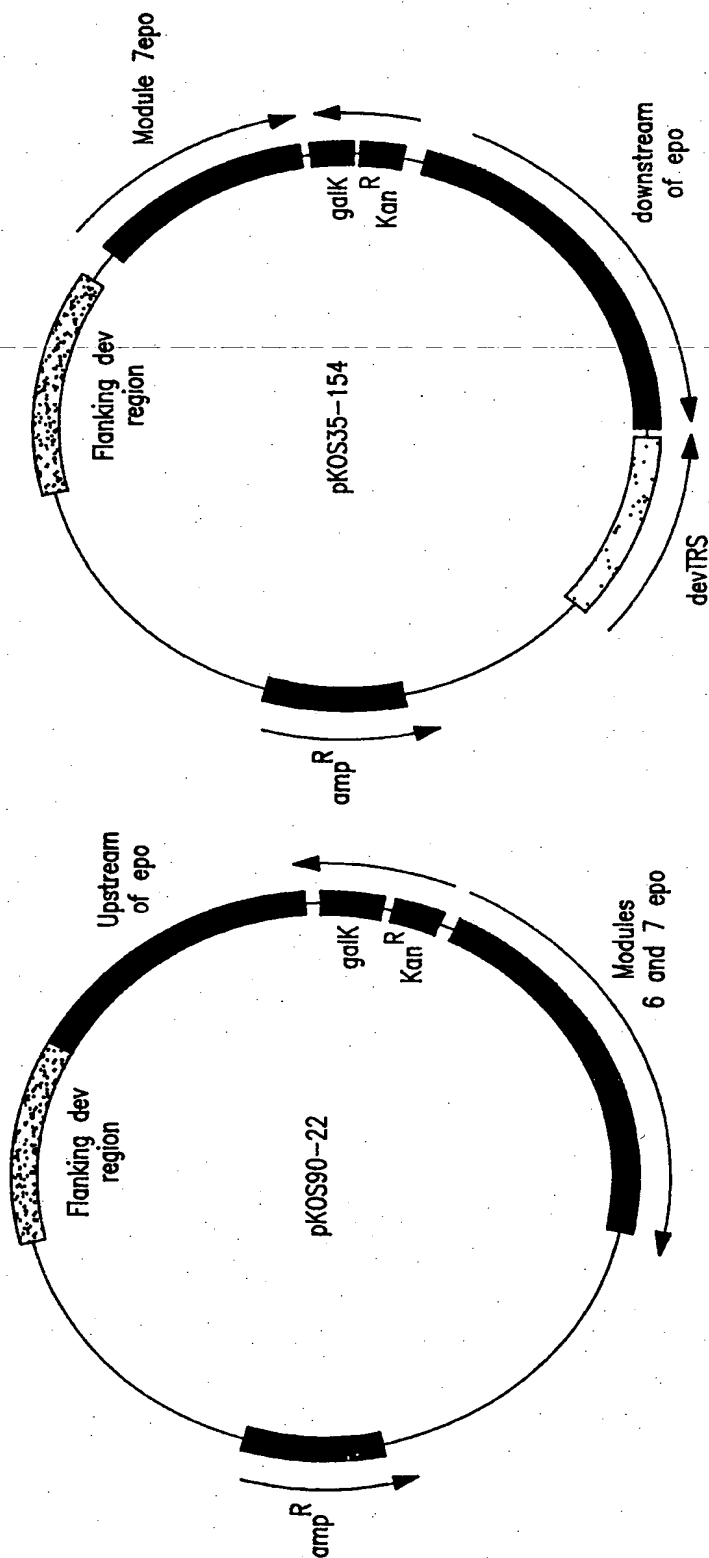


FIG. 4

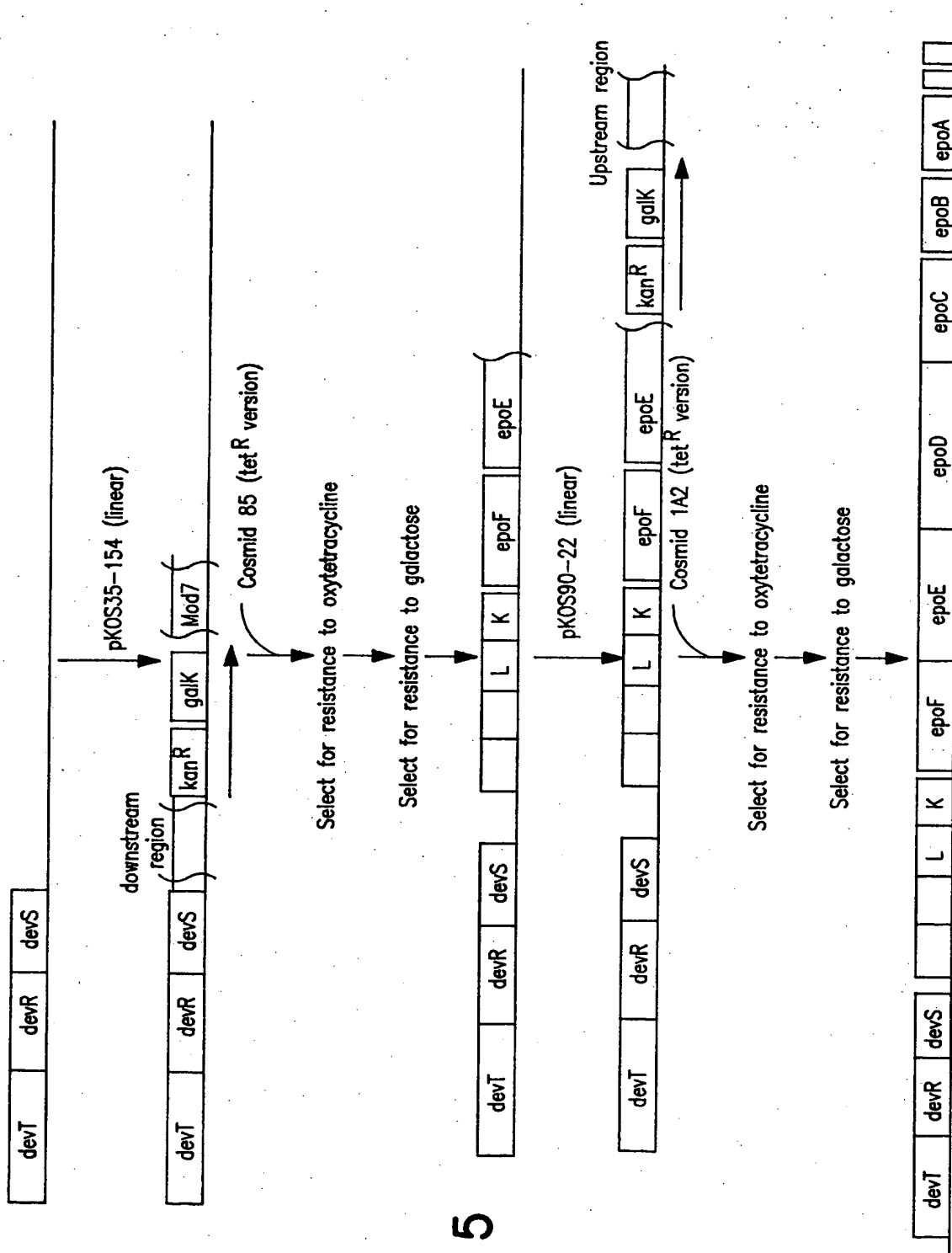


FIG. 5

6/8

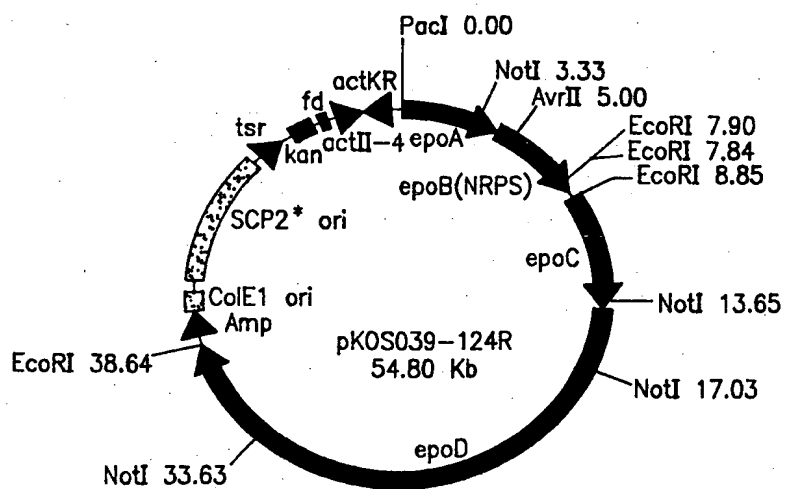
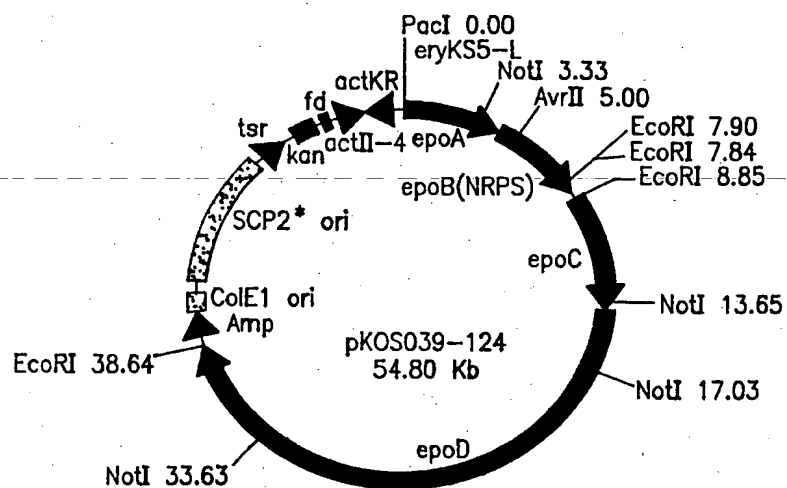


FIG. 6



7/8

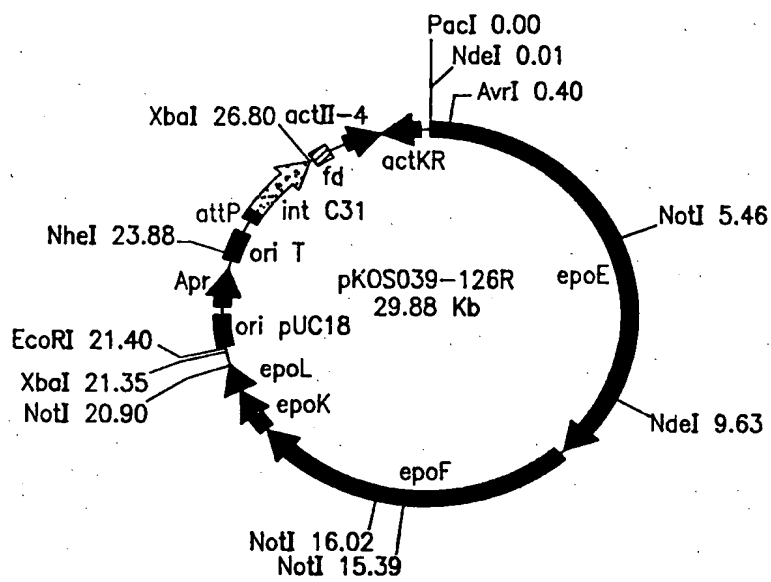


FIG. 7

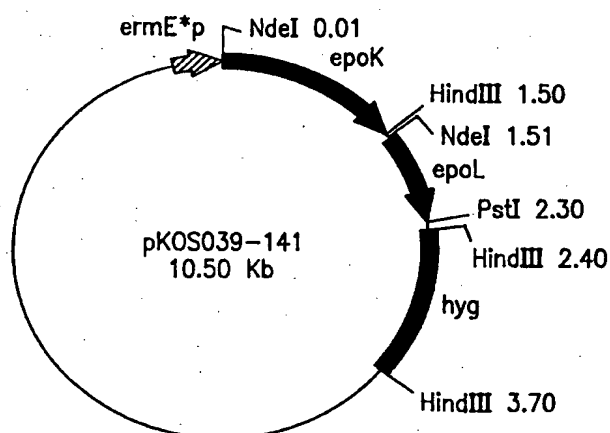


FIG. 8

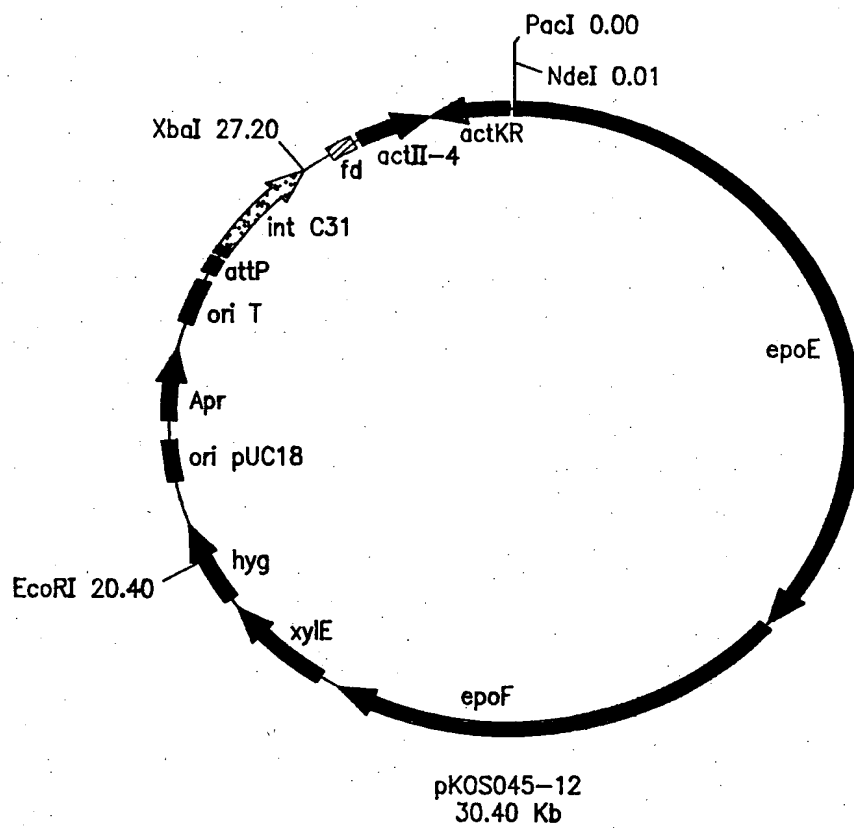


FIG. 9

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
2 June 2000 (02.06.2000)

(10) International Publication Number  
**WO 00/31247 A3**

PCT

- (51) International Patent Classification<sup>7</sup>: C12N 9/00, 15/52, C12P 17/06
- (21) International Application Number: PCT/US99/27438
- (22) International Filing Date:  
19 November 1999 (19.11.1999)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
60/109,401 20 November 1998 (20.11.1998) US  
60/119,386 10 February 1999 (10.02.1999) US  
60/122,620 3 March 1999 (03.03.1999) US  
60/130,560 22 April 1999 (22.04.1999) US
- (71) Applicant: KOSAN BIOSCIENCES, INC. [US/US];  
3832 Bay Center Drive, Hayward, CA 94545 (US).
- (72) Inventors: JULIEN, Bryan; 4514 Fleming Avenue,  
Oakland, CA 94619 (US). KATZ, Leonard; 22294 City  
Center Drive, Apartment 5104, Hayward, CA 95051 (US).  
KHOSLA, Chaitan; 740 La Para Avenue, Palo Alto,  
CA 94306 (US). TANG, Li; 574 Cutwater Lane, Foster  
City, CA 94404 (US). ZIERMANN, Rainer; 3908 Leona  
Street, San Mateo, CA 94403 (US).
- (54) Title: RECOMBINANT METHODS AND MATERIALS FOR PRODUCING EPOTHILONE AND EPOTHILONE DERIVATIVES
- (57) Abstract: Recombinant nucleic acids that encode all or a portion of the epothilone polyketide synthase (PKS) of *Sorangium cellulosum* are used to express recombinant PKS genes in host cells for the production of epothilones, epothilone derivatives, and polyketides that are useful as cancer chemotherapeutics, fungicides, and immunosuppressants.
- (74) Agents: MURASHIGE, Kate, H. et al.; Morrison & Foerster LLP, 2000 Pennsylvania Avenue, N.W., Washington, DC 20006-1888 (US).
- (81) Designated States (*national*): AL, AM, AU, BA, BB, BG, BR, CA, CN, CR, CU, CZ, DM, EE, GD, GE, HR, HU, IL, IS, JP, KG, KP, KR, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, UZ, VN, ZA.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:  
— With international search report.
- (88) Date of publication of the international search report:  
7 December 2000
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*



WO 00/31247 A3

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 99/27438

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N9/00 C12N15/52 C12P17/06

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 99 66028 A (NOVARTIS ERFIND VERWALT GMBH ;NOVARTIS AG (CH); SCHUPP THOMAS (CH)) 23 December 1999 (1999-12-23) the whole document ---	1-22,24, 28
E	WO 00 22139 A (BIOTECHNOLOG FORSCHUNG GMBH) 20 April 2000 (2000-04-20) the whole document ---	1-22,24, 28
T	MOLNÁR I. ET AL.: "The biosynthetic gene cluster for ... epothilones A and B from Sorangium cellulosum So ce90." CHEMISTRY & BIOLOGY VOL 7 NO 2, 5 January 2000 (2000-01-05), pages 97-109, XP000904734 the whole document ---	1-22,24, 28
	-/-	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*Z\* document member of the same patent family

Date of the actual completion of the international search

23 June 2000

Date of mailing of the international search report

14.09.2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Hardon, E

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 99/27438

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	TANG, L. ET AL.: "Cloning and heterologous expression of the epithilone gene cluster." SCIENCE, vol. 287, 28 January 2000 (2000-01-28), pages 640-42, XP002135341 the whole document	1-22,24, 28
A	WO 98 22461 A (BIOTECHNOLOG FORSCHUNG GMBH ;GERTH KLAUS (DE); HOEFLE GERHARD (DE)) 28 May 1998 (1998-05-28) the whole document	1-22,24, 28
A	CHOU T. C. ET AL.: "DESOXYEPITHILONE B: AN EFFICACIOUS MICROTUBULE-TARGETED ANTITUMOR AGENT WITH A PROMISING IN VIVO PROFILE RELATIVE TO EPOTHILONE B." PROC. NATL. ACAD. SCI. USA, vol. 95, no. 16, 4 August 1998 (1998-08-04), pages 9642--7, XP000910107 the whole document	1-22,24, 28

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 99/27438

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-22, 24, 28

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-22, 24, 28

nucleic acid encoding at least a domain of an epothilone  
polyketide synthase and/or modification enzyme.

2. Claims: 23, 25-27

epothilone compounds (16 inventions).

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/27438

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9966028	A	23-12-1999	AU	4611699 A	05-01-2000
WO 0022139	A	20-04-2000	DE	19846493 A	13-04-2000
			AU	6512699 A	01-05-2000
WO 9822461	A	28-05-1998	AU	5483798 A	10-06-1998
			BR	9713363 A	25-01-2000
			CN	1237970 A	08-12-1999
			CZ	9901750 A	15-09-1999
			EP	0941227 A	15-09-1999
			NO	992338 A	14-05-1999
			PL	333435 A	06-12-1999
			ZA	9710384 A	18-05-1999



**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**

---

**THIS PAGE BLANK (USPTO)**